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(54) Title: REGULATION OF HUMAN ESTERASE

(57) Abstract: Reagents that regulate human esterase and reagents which bind to human esterase gene products can play a role in preventing, ameliorating, or correcting dysfunctions or diseases including, but not limited to, cancer, dermatological disorders, gastrointestinal and liver disorders, neurological disorders, respiratory disorders, metabolic disorders, inflammatory disorders, reproductive disorders, and genitourinary disorders.

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REGULATION OF HUMAN ESTERASE

This application incorporates by reference co-pending provisional applications Serial No. 60/399,432 filed July 31, 2002, Serial No. 60/430,667 filed December 4, 2002, and Serial No. 60/459,984 filed April 4, 2003.

FIELD OF THE INVENTION

The invention relates to the regulation of human esterase.

BACKGROUND OF THE INVENTION

There is a need in the art to identify esterases, which can be regulated to provide therapeutic effects.

It is an object of the invention to provide reagents and methods of regulating a human esterase. This and other objects of the invention are provided by one or more of the embodiments described below.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 1).

Fig. 2 shows the amino acid sequence deduced from the DNA-sequence of Fig.1 (SEQ ID NO: 2).

Fig. 3 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 3).

Fig. 4 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 4).

Fig. 5 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 5).

Fig. 6 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 6).

Fig. 7 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 7).

Fig. 8 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 8).

Fig. 9 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 9).

Fig. 10 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 10).

Fig. 11 shows the DNA-sequence encoding an esterase Polypeptide (SEQ ID NO: 11).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to an isolated polynucleotide from the group consisting of:

a) a polynucleotide encoding an Esterase polypeptide comprising an amino acid sequence selected from the group consisting of:
amino acid sequences which are at least about 97% identical to the amino acid sequence shown in SEQ ID NO: 2; and
the amino acid sequence shown in SEQ ID NO: 2.

b) a polynucleotide comprising the sequence of SEQ ID NO: 1;

c) a polynucleotide which hybridizes under stringent conditions to a polynucleotide specified in (a) and (b) and encodes a Esterase polypeptide;

d) a polynucleotide the sequence of which deviates from the polynucleotide sequences specified in (a) to (c) due to the degeneration of the genetic code and encodes a Esterase polypeptide; and

e) a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d) and encodes a Esterase polypeptide.

A novel human esterase is a discovery of the present invention. Human esterase comprises the amino acid sequence shown in SEQ ID NO: 2. A coding sequence for human esterase is shown in SEQ ID NO: 1. This sequence is contained within the longer sequence shown in SEQ ID NO: 3. This sequence is located on chromosome 15q25.3. Related ESTs (SEQ ID NOS: 4-11) are expressed in brain, colon, pancreas, epithelioid carcinoma, stomach and liver cells.

The esterase of the invention is associated with a general family of alpha/beta hydrolases and, more specifically, to a group of esterases designated phopholipase/-carboxylesterase. Furthermore, an alpha/beta hydrolase domain and the esterase family active site are found in the esterase of the invention. The esterase protein of the invention contains three active site amino acid residues identified in carboxylesterase as involved in the charge relay system.

Human esterase of the invention is expected to be useful for the same purposes as previously identified esterase enzymes. Human esterase is believed to be useful in therapeutic methods to treat disorders such as cancer, dermatological disorders, gastrointestinal and liver disorders, neurological disorders, respiratory disorders, metabolic disorders, inflammatory disorders, reproductive disorders, and genitourinary disorders. Human esterase also can be used to screen for human esterase activators and inhibitors.

One embodiment of the present invention is an expression vector containing any polynucleotide of the present invention.

Yet another embodiment of the present invention is a host cell containing any expression vector of the present invention.

Still another embodiment of the present invention is a substantially purified Esterase polypeptide encoded by any polynucleotide of the present invention.

Even another embodiment of the present invention is a method of producing a Esterase polypeptide of the present invention, wherein the method comprises the following steps:

a. culturing the host cells of the present invention under conditions suitable for the expression of the Esterase polypeptide; and

b. recovering the Esterase polypeptide from the host cell culture.

Yet another embodiment of the present invention is a method for detecting a polynucleotide encoding a Esterase polypeptide in a biological sample comprising the following steps:

a. hybridizing any polynucleotide of the present invention to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and

b. detecting said hybridization complex.

Still another embodiment of the present invention is a method for detecting a polynucleotide of the present invention or a Esterase polypeptide of the present invention comprising the steps of:

a. contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the Esterase polypeptide and

b. detecting the interaction

Even another embodiment of the present invention is a diagnostic kit for conducting any method of the present invention.

Yet another embodiment of the present invention is a method of screening for agents which decrease the activity of a Esterase, comprising the steps of:

a. contacting a test compound with a Esterase polypeptide encoded by any polynucleotide of the present invention;

b. detecting binding of the test compound to the Esterase polypeptide, wherein a test compound which binds to the polypeptide is identified as a potential therapeutic agent for decreasing the activity of a Esterase.

Still another embodiment of the present invention is a method of screening for agents which regulate the activity of a Esterase, comprising the steps of:

a. contacting a test compound with a Esterase polypeptide encoded by any polynucleotide of the present invention; and

b. detecting a Esterase activity of the polypeptide, wherein a test compound which increases the Esterase activity is identified as a potential therapeutic agent for increasing the activity of the Esterase, and wherein a test compound which decreases the Esterase activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the Esterase.

Even another embodiment of the present invention is a method of screening for agents which decrease the activity of a Esterase, comprising the step of:

contacting a test compound with any polynucleotide of the present invention and detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of Esterase.

Yet another embodiment of the present invention is a method of reducing the activity of a Esterase, comprising the step of:

contacting a cell with a reagent which specifically binds to any polynucleotide of the present invention or any Esterase polypeptide of the present invention, whereby the activity of Esterase is reduced.

Still another embodiment of the present invention is a reagent that modulates the activity of a Esterase polypeptide or a polynucleotide wherein said reagent is identified by any methods of the present invention.

Even another embodiment of the present invention is a pharmaceutical composition, comprising:

an expression vector of the present invention or a reagent of the present invention and a pharmaceutically acceptable carrier.

Yet another embodiment of the present invention is the use of an expression vector of the present invention or a reagent of the present invention for modulating the activity of a Esterase in a disease, preferably cancer, a dermatological disorder, a gastro-intestinal and a liver disorder, a neurological disorder, a respiratory disorder, a metabolic disorder, an inflammatory disorder, a reproductive disorder or a genitou-rinary disorder.

The invention thus provides a human esterase that can be used to identify test compounds that may act, for example, as activators or inhibitors at the enzyme's active site. Human esterase and fragments thereof also are useful in raising specific antibodies that can block the enzyme and effectively reduce its activity.

Polypeptides

Human esterase polypeptides according to the invention comprise at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, or 329 contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof, as defined below. An esterase polypeptide of the invention therefore can be a portion of an esterase protein, a full-length esterase protein, or a fusion protein comprising all or a portion of an esterase protein.

Biologically active variants

Human esterase polypeptide variants that are biologically active, *e.g.*, retain an enzymatic activity, also are human esterase polypeptides. Preferably, naturally or non-naturally occurring human esterase polypeptide variants have amino acid sequences which are at least about 92, preferably about 95, 96, 97, 98, or 99% identical to the amino acid sequence shown in SEQ ID NO: 2 or a fragment thereof. Percent identity between a putative human esterase polypeptide variant and an amino acid sequence of SEQ ID NO: 2 is determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio. 48*:603 (1986), and Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA 89*:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff & Henikoff, 1992.

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson & Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative variant. The FASTA algorithm is described by Pearson & Lipman, *Proc. Nat'l Acad. Sci. USA 85*:2444(1988), and by Pearson, *Meth. Enzymol. 183*:63 (1990). Briefly, FASTA first characterizes sequence

similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO: 2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch- Sellers algorithm (Needleman & Wunsch, *J. Mol. Biol.48*:444 (1970); Sellers, *SIAM J. Appl. Math.26*:787 (1974)), which allows for amino acid insertions and deletions. Preferred parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol. 183*:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as default.

"% identity" of a first sequence towards a second sequence, within the meaning of the invention, shall be understood as being the % identity which is calculated as follows: First the optimal global alignment between the two sequences is determined with the CLUSTAL W algorithm [Thomson JD, Higgins DG, gibson TJ. 1994. ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix

choice. Nuleic Acids Res., 22: 4673-4680], Version 1.8, applying the following command line syntax: ./clustalw -infile=./infile.txt -output= -outorder=aligned -pwmatrix=gonnet -pwdnamatrix=clustalw -pwgapopen=10.0 -pwgapext=0.1 -matrix=gonnet -gapopen=10.0 -gapext=0.05 -gapdist=8 -hgapresidues=GPSNDQERK -maxdiv=40. (Implementations of the CLUSTAL W algorithm are readily available at numerous sites on the internet, including, e.g., http://www.ebi.ac.uk.) Thereafter, the number of matches in the alignment is determined by counting the number of identical nucleotides (or amino acid residues) in aligned positions. Finally, the total number of matches is divided by the total number of nucleotides (or amino acid residues) of the longer of the two sequences; and multiplied by 100 to yield the "% identity" of a first sequence towards a second sequence.

Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine. Polypeptides of the invention may have, for example, between 1 and 10 (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) conservative amino acid substitutions.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of a human esterase polypeptide can be found using computer programs well known in the art, such as DNASTAR software.

The invention additionally, encompasses esterase polypeptides that are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Any of numerous chemical modifications can be carried out by known techniques including, but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of prokaryotic host cell expression. The esterase polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

The invention also provides chemically modified derivatives of esterase polypeptides that may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization can be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol, and the like. The polypeptides can be modified at random or predetermined positions within the molecule and can include one, two, three, or more attached chemical moieties.

Whether an amino acid change or a polypeptide modification results in a biologically active esterase polypeptide can readily be determined by assaying for enzymatic activity, as described in the specific examples, below.

Fusion proteins

Fusion proteins are useful for generating antibodies against esterase polypeptide amino acid sequences and for use in various assay systems. For example, fusion

proteins can be used to identify proteins that interact with portions of a human esterase polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

A human esterase polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment comprises at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, or 329 contiguous amino acids of SEQ ID NO:2 or of a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length esterase protein.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include β-galactosidase, β-glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horse-radish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the esterase polypeptide-encoding sequence and the heterologous protein sequence, so that the esterase polypeptide can be cleaved and purified away from the heterologous moiety.

A fusion protein can be synthesized chemically, as is known in the art. Preferably, a fusion protein is produced by covalently linking two polypeptide segments or by standard procedures in the art of molecular biology. Recombinant DNA methods can

be used to prepare fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from SEQ ID NO:1 in proper reading frame with nucleotides encoding the second polypeptide segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International Corporation (MIC; Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

Identification of species homologs

Species homologs of human esterase polypeptide can be obtained using esterase polypeptide polynucleotides (described below) to make suitable probes or primers for screening cDNA expression libraries from other species, such as mice, monkeys, or yeast, identifying cDNAs which encode homologs of esterase polypeptide, and expressing the cDNAs as is known in the art.

Polynucleotides

A human esterase polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for an esterase polypeptide. A coding sequence for human esterase is shown in SEQ ID NO: 1.

Degenerate nucleotide sequences encoding human esterase polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 70, preferably about 75, 90, 96, 98, or 99% identical to the nucleotide sequence shown in SEQ ID NO: 1 or its complement also are esterase polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of esterase

polynucleotides that encode biologically active esterase polypeptides also are esterase polynucleotides. Polynucleotide fragments comprising at least 8, 9, 10, 11, 12, 15, 20, or 25 contiguous nucleotides of SEQ ID NO:1 or its complement also are esterase polynucleotides. These fragments can be used, for example, as hybridization probes or as antisense oligonucleotides.

Identification of polynucleotide variants and homologs

Variants and homologs of the esterase polynucleotides described above also are esterase polynucleotides. Typically, homologous esterase polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known esterase polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions--2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50°C once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes each--homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the esterase polynucleotides disclosed herein also can be identified by making suitable probes or primers and screening cDNA expression libraries from other species, such as mice, monkeys, or yeast. Human variants of esterase polynucleotides can be identified, for example, by screening human cDNA expression libraries. It is well known that the T_m of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner *et al.*, *J. Mol. Biol. 81*, 123 (1973). Variants of human esterase polynucleotides or esterase polynucleotides of other species can therefore be identified by hybridizing a putative homologous esterase polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID NO: 1 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary nucleotide

sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

Nucleotide sequences which hybridize to esterase polynucleotides or their complements following stringent hybridization and/or wash conditions also are esterase polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50–9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20°C below the calculated T_m of the hybrid under study. The T_m of a hybrid between an esterase polynucleotide having a nucleotide sequence shown in SEQ ID NO: 1 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A. 48*, 1390 (1962):

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - 0.63(\%\text{formamide}) - 600/l),$$

where l = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65°C, or 50% formamide, 4X SSC at 42°C, or 0.5X SSC, 0.1% SDS at 65°C. Highly stringent wash conditions include, for example, 0.2X SSC at 65°C.

Preparation of polynucleotides

A human esterase polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Polynucleotides can be made by a cell and isolated using standard nucleic acid purification techniques, or synthesized using an amplification technique, such as the polymerase chain reaction (PCR), or by

using an automatic synthesizer. Methods for isolating polynucleotides are routine and are known in the art. Any such technique for obtaining a polynucleotide can be used to obtain isolated esterase polynucleotides. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments, which comprise esterase nucleotide sequences. Isolated polynucleotides are in preparations that are free or at least 70, 80, or 90% free of other molecules.

Human esterase cDNA molecules can be made with standard molecular biology techniques, using esterase mRNA as a template. Human esterase cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook *et al.* (1989). An amplification technique, such as PCR, can be used to obtain additional copies of polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesize esterase polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a human esterase polypeptide having, for example, an amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof.

Extending polynucleotides

Various PCR-based methods can be used to extend the nucleic acid sequences disclosed herein to detect upstream sequences such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus. Sarkar, *PCR Methods Applic. 2*, 318-322, 1993; Triglia *et al.*, *Nucleic Acids Res. 16*, 8186, 1988; Lagerstrom *et al.*, *PCR Methods Applic. 1*, 111-119, 1991; Parker *et al.*, *Nucleic Acids Res. 19*, 3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to walk genomic DNA (CLONTECH, Palo Alto, Calif). See WO 01/98340.

Obtaining Polynucleotides

Human esterase polypeptides can be obtained, for example, by purification from human cells, by expression of esterase polynucleotides, or by direct chemical synthesis.

Protein purification

Human esterase polypeptides can be purified from any human cell which expresses the receptor, including host cells which have been transfected with esterase polynucleotides. A purified esterase polypeptide is separated from other compounds that normally associate with the esterase polypeptide in the cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis.

A preparation of purified esterase polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

Expression of polynucleotides

To express a human esterase polynucleotide, the polynucleotide can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding esterase polypeptides and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are

described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

A variety of expression vector/host systems can be used to contain and express sequences encoding a human esterase polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems. See WO 01/98340.

Host cells

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed esterase polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells that have specific cellular machinery and characteristic mechanisms for post-translational activities (*e.g.*, CHO, HeLa, MDCK, HEK293, and WI38) are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein. See WO 01/98340.

Alternatively, host cells which contain a human esterase polynucleotide and which express a human esterase polypeptide can be identified by a variety of procedures known to those of skill in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). Hampton *et al.*, SEROLOGICAL METHODS: A LABORATORY MANUAL, APS

Press, St. Paul, Minn., 1990) and Maddox *et al.*, *J. Exp. Med. 158*, 1211-1216, 1983). See WO 01/98340.

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding esterase polypeptides include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a human esterase polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Expression and purification of polypeptides

Host cells transformed with nucleotide sequences encoding a human esterase polypeptide can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode esterase polypeptides can be designed to contain signal sequences which direct secretion of soluble esterase polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound esterase polypeptide. See WO 01/98340.

Chemical synthesis

Sequences encoding a human esterase polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al. Nucl. Acids Res. Symp. Ser.* 225-232, 1980). Alternatively, a human esterase polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc. 85*, 2149-2154, 1963; Roberge *et al.*, *Science 269*, 202-204, 1995). Protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of esterase polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule. See WO 01/98340.

As will be understood by those of skill in the art, it may be advantageous to produce esterase polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter esterase polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

Antibodies

Any type of antibody known in the art can be generated to bind specifically to an epitope of a human esterase polypeptide. "Antibody" as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, $F(ab')_2$, and Fv, which are capable of binding an epitope of a human esterase polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, *e.g.*, at least 15, 25, or 50 amino acids.

An antibody which specifically binds to an epitope of a human esterase polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody that specifically binds to the immunogen.

Typically, an antibody that specifically binds to a human esterase polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, antibodies that specifically bind to esterase polypeptides do not detect other proteins in immunochemical assays and can immunoprecipitate a human esterase polypeptide from solution. See WO 01/98340.

Antisense oligonucleotides

Antisense oligonucleotides are nucleotide sequences that are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of esterase gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. *See* Brown, *Meth. Mol. Biol. 20*, 1-8, 1994; Sonveaux, *Meth. Mol. Biol. 26*, 1-72, 1994; Uhlmann *et al.*, *Chem. Rev. 90*, 543-583, 1990.

Modifications of esterase gene expression can be obtained by designing antisense oligonucleotides that will form duplexes to the control, 5', or regulatory regions of the esterase gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be

designed to block translation of mRNA by preventing the transcript from binding to ribosomes. See WO 01/98340.

Ribozymes

Ribozymes are RNA molecules with catalytic activity. *See, e.g.*, Cech, *Science 236*, 1532-1539; 1987; Cech, *Ann. Rev. Biochem. 59*, 543-568; 1990, Cech, *Curr. Opin. Struct. Biol. 2*, 605-609; 1992, Couture & Stinchcomb, *Trends Genet. 12*, 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (*e.g.*, Haseloff *et al.*, U.S. Patent 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

The coding sequence of a human esterase polynucleotide can be used to generate ribozymes that will specifically bind to mRNA transcribed from the esterase polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (*see* Haseloff *et al. Nature 334*, 585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.*, EP 321,201). See WO 01/98340.

Differentially expressed genes

Described herein are methods for the identification of genes whose products interact with human esterase. Such genes may represent genes that are differentially

expressed in disorders including, but not limited to, cancer, dermatological disorders, gastrointestinal and liver disorders, neurological disorders, respiratory disorders, metabolic disorders, inflammatory disorders, reproductive disorders, and genitourinary disorders. Further, such genes may represent genes that are differentially regulated in response to manipulations relevant to the progression or treatment of such diseases. Additionally, such genes may have a temporally modulated expression, increased or decreased at different stages of tissue or organism development. A differentially expressed gene may also have its expression modulated under control versus experimental conditions. In addition, the human esterase gene or gene product may itself be tested for differential expression.

The degree to which expression differs in a normal versus a diseased state need only be large enough to be visualized via standard characterization techniques such as differential display techniques. Other such standard characterization techniques by which expression differences may be visualized include but are not limited to, quantitative RT (reverse transcriptase), PCR, and Northern analysis.

To identify differentially expressed genes total RNA or, preferably, mRNA is isolated from tissues of interest. For example, RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects. Any RNA isolation technique that does not select against the isolation of mRNA may be utilized for the purification of such RNA samples. See, for example, Ausubel *et al.*, ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc. New York, 1987-1993. Large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, U.S. Patent 4,843,155.

Transcripts within the collected RNA samples that represent RNA produced by differentially expressed genes are identified by methods well known to those of skill in the art. They include, for example, differential screening (Tedder *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 85*, 208-12, 1988), subtractive hybridization (Hedrick *et al.*,

Nature 308, 149-53; Lee *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 88*, 2825, 1984), and, preferably, differential display (Liang & Pardee, *Science 257*, 967-71, 1992; U.S. Patent 5,262,311).

The differential expression information may itself suggest relevant methods for the treatment of disorders involving the human esterase. For example, treatment may include a modulation of expression of the differentially expressed genes and/or the gene encoding the human esterase. The differential expression information may indicate whether the expression or activity of the differentially expressed gene or gene product or the human esterase gene or gene product are up-regulated or down-regulated.

Screening methods

The invention provides assays for screening test compounds that bind to or modulate the activity of a human esterase polypeptide or a human esterase polynucleotide. A test compound preferably binds to a human esterase polypeptide or polynucleotide. More preferably, a test compound decreases or increases enzymatic activity by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

Test compounds

Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods

requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. *See* Lam, *Anticancer Drug Des. 12*, 145, 1997.

Methods for the synthesis of molecular libraries are well known in the art (*see*, for example, DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6909, 1993; Erb *et al. Proc. Natl. Acad. Sci. U.S.A. 91*, 11422, 1994; Zuckermann *et al.*, *J. Med. Chem. 37*, 2678, 1994; Cho *et al.*, *Science 261*, 1303, 1993; Carell *et al.*, *Angew. Chem. Int. Ed. Engl. 33*, 2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl. 33*, 2061; Gallop *et al.*, *J. Med. Chem. 37*, 1233, 1994). Libraries of compounds can be presented in solution (*see, e.g.*, Houghten, *BioTechniques 13*, 412-421, 1992), or on beads (Lam, *Nature 354*, 82-84, 1991), chips (Fodor, *Nature 364*, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 89*, 1865-1869, 1992), or phage (Scott & Smith, *Science 249*, 386-390, 1990; Devlin, *Science 249*, 404-406, 1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci. 97*, 6378-6382, 1990; Felici, *J. Mol. Biol. 222*, 301-310, 1991; and Ladner, U.S. Patent 5,223,409).

High through-put screening

Test compounds can be screened for the ability to bind to esterase polypeptides or polynucleotides or to affect esterase activity or esterase gene expression using high throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500 µl. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format.

Alternatively, "free format assays," or assays that have no physical barrier between samples, can be used. For example, an assay using pigment cells (melanocytes) in a simple homogeneous assay for combinatorial peptide libraries is described by Jayawickreme *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 19,* 1614-18 (1994). The cells are placed under agarose in petri dishes, then beads that carry combinatorial compounds are placed on the surface of the agarose. The combinatorial compounds are partially released the compounds from the beads. Active compounds can be visualized as dark pigment areas because, as the compounds diffuse locally into the gel matrix, the active compounds cause the cells to change colors.

Another example of a free format assay is described by Chelsky, "Strategies for Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at the First Annual Conference of The Society for Biomolecular Screening in Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel would cause a color change throughout the gel. Thereafter, beads carrying combinatorial compounds via a photolinker were placed inside the gel and the compounds were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

Yet another example is described by Salmon *et al.*, *Molecular Diversity 2,* 57-63 (1996). In this example, combinatorial libraries were screened for compounds that had cytotoxic effects on cancer cells growing in agar.

Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more assay components are then placed within, on top of, or at the bottom of a matrix such as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support. When samples are introduced to the porous matrix they diffuse sufficiently slowly, such that the assays can be performed without the test samples running together.

Binding assays

For binding assays, the test compound is preferably a small molecule that binds to and occupies, for example, the active site of the esterase polypeptide, such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules.

In binding assays, either the test compound or the esterase polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound that is bound to the esterase polypeptide can then be accomplished, for example, by direct counting of radioemmission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

Alternatively, binding of a test compound to a human esterase polypeptide can be determined without labeling either of the interactants. For example, a microphysiometer can be used to detect binding of a test compound with a human esterase polypeptide. A microphysiometer (*e.g.*, Cytosensor™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and a human esterase polypeptide (McConnell *et al.*, *Science 257*, 1906-1912, 1992).

Determining the ability of a test compound to bind to a human esterase polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, *Anal. Chem. 63*, 2338-2345, 1991, and Szabo *et al.*, *Curr. Opin. Struct. Biol. 5*, 699-705, 1995). BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore™). Changes in the optical phenomenon surface

plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another aspect of the invention, a human esterase polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent 5,283,317; Zervos *et al.*, *Cell 72*, 223-232, 1993; Madura *et al.*, *J. Biol. Chem. 268*, 12046-12054, 1993; Bartel *et al.*, *BioTechniques 14*, 920-924, 1993; Iwabuchi *et al.*, *Oncogene 8*, 1693-1696, 1993; and Brent WO94/10300), to identify other proteins which bind to or interact with the esterase polypeptide and modulate its activity.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. For example, in one construct, polynucleotide encoding a human esterase polypeptide can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo* to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ), which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the DNA sequence encoding the protein that interacts with the esterase polypeptide.

It may be desirable to immobilize either the esterase polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the esterase polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass

or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach the enzyme polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to a human esterase polypeptide (or polynucleotide) can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

In one embodiment, the esterase polypeptide is a fusion protein comprising a domain that allows the esterase polypeptide to be bound to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed esterase polypeptide; the mixture is then incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a human esterase polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated esterase polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS(N-hydroxysuccinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated

96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to an esterase polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of the esterase polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the esterase polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the esterase polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a human esterase polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises an esterase polypeptide or polynucleotide can be used in a cell-based assay system. An esterase polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to an esterase polypeptide or polynucleotide is determined as described above.

Enzymatic activity

Test compounds can be tested for the ability to increase or decrease the enzymatic activity of a human esterase polypeptide. Enzymatic activity can be measured, for example, as described in Example 4.

Enzyme assays can be carried out after contacting either a purified esterase polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound that decreases enzymatic activity of a human esterase polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for decreasing esterase activity. A test compound which increases enzymatic activity of a human esterase polypeptide by at

least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for increasing human esterase activity.

Gene expression

In another embodiment, test compounds that increase or decrease esterase gene expression are identified. An esterase polynucleotide is contacted with a test compound, and the expression of an RNA or polypeptide product of the esterase polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the mRNA or polypeptide expression.

The level of esterase mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of polypeptide products of a human esterase polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunochemical methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro* translation system by detecting incorporation of labeled amino acids into a human esterase polypeptide.

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell that expresses a human esterase polynucleotide can be used in a cell-

based assay system. The esterase polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Either a primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

Pharmaceutical compositions

The invention also provides pharmaceutical compositions that can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a human esterase polypeptide, esterase polynucleotide, ribozymes or antisense oligonucleotides, antibodies which specifically bind to an esterase polypeptide, or mimetics, activators, or inhibitors of a human esterase polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, *i.e.*, dosage.

Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions can contain substances that increase the viscosity of the

suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers also can be used for delivery. Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention can be manufactured in a manner that is known in the art, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

Therapeutic indications and methods

Human esterase can be regulated to treat cancer, dermatological disorders, gastro-intestinal and liver disorders, neurological disorders, respiratory disorders, metabolic disorders, inflammatory disorders, reproductive disorders, and genitourinary disorders.

Cancer

The human esterase of the invention is highly expressed in the following cancer tissues: esophageal tumor, stomach tumor, colon tumor, ileum tumor, liver tumor, HEP G2 cells, glial tumor H4 cells, lung tumor, uterus tumor, ovary tumor, breast tumor, MDA MB 231 cells (breast tumor), and prostate. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue and healthy tissue demonstrates that the esterase protein or mRNA can be used to diagnose cancer. In addition, the activity of the esterase protein can be modulated to treat cancer.

Cancer is a disease fundamentally caused by oncogenic cellular transformation. There are several hallmarks of transformed cells that distinguish them from their normal counterparts and underlie the pathophysiology of cancer. These include uncontrolled cellular proliferation, unresponsiveness to normal death-inducing signals (immortalization), increased cellular motility and invasiveness, increased ability to recruit blood supply through induction of new blood vessel formation (angiogenesis), genetic instability, and dysregulated gene expression. Various combinations of these aberrant physiologies, along with the acquisition of drug-resistance frequently lead to an intractable disease state in which organ failure and patient death ultimately ensue.

Cancer disorders within the scope of the invention comprise any disease of an organ or tissue in mammals characterized by poorly controlled or uncontrolled

multiplication of normal or abnormal cells in that tissue and its effect on the body as a whole. Cancer diseases within the scope of the invention comprise benign neoplasms, dysplasias, hyperplasias as well as neoplasms showing metastatic growth or any other transformations, *e.g.*, leukoplakias, which often precede a breakout of cancer. Cells and tissues are cancerous when they grow more rapidly than normal cells, displacing or spreading into the surrounding healthy tissue or any other tissues of the body described as metastatic growth, assume abnormal shapes and sizes, show changes in their nucleocytoplasmatic ratio, nuclear polychromasia, and finally may cease.

Cancerous cells and tissues may affect the body as a whole when causing paraneoplastic syndromes or if cancer occurs within a vital organ or tissue, normal function will be impaired or halted, with possible fatal results. The ultimate involvement of a vital organ by cancer, either primary or metastatic, may lead to the death of the mammal affected. Cancer tends to spread, and the extent of its spread is usually related to an individual's chances of surviving the disease. Cancers are generally said to be in one of three stages of growth: early, or localized, when a tumor is still confined to the tissue of origin, or primary site; direct extension, where cancer cells from the tumour have invaded adjacent tissue or have spread only to regional lymph nodes; or metastasis, in which cancer cells have migrated to distant parts of the body from the primary site, via the blood or lymph systems, and have established secondary sites of infection. Cancer is said to be malignant because of its tendency to cause death if not treated.

Benign tumors usually do not cause death, although they may if they interfere with a normal body function by virtue of their location, size, or paraneoplastic side effects. Hence, benign tumors fall under the definition of cancer within the scope of the invention as well. In general, cancer cells divide at a higher rate than do normal cells, but the distinction between the growth of cancerous and normal tissues is not so much the rapidity of cell division in the former as it is the partial or complete loss of growth restraint in cancer cells and their failure to differentiate into a useful, limited

tissue of the type that characterizes the functional equilibrium of growth of normal tissue.

Cancer tissues may express certain molecular receptors and probably are influenced by the host's susceptibility and immunity and it is known that certain cancers of the breast and prostate, for example, are considered dependent on specific hormones for their existence. The term "cancer" under the scope of the invention is not limited to simple benign neoplasia but includes any other benign and malign neoplasia, such as 1) carcinoma, 2) sarcoma, 3) carcinosarcoma, 4) cancers of the blood-forming tissues, 5) tumors of nerve tissues including the brain, and 6) cancer of skin cells.

Carcinoma occurs in epithelial tissues, which cover the outer body (the skin) and line mucous membranes and the inner cavitary structures of organs e.g. such as the breast, lung, the respiratory and gastrointestinal tracts, the endocrine glands, and the genitourinary system. Ductal or glandular elements may persist in epithelial tumors, as in adenocarcinomas, *e.g.*, thyroid adenocarcinoma, gastric adenocarcinoma, uterine adenocarcinoma. Cancers of the pavement-cell epithelium of the skin and of certain mucous membranes, such as cancers of the tongue, lip, larynx, urinary bladder, uterine cervix, or penis, may be termed epidermoid or squamous-cell carcinomas of the respective tissues and are within the scope of the definition of cancer as well.

Sarcomas develop in connective tissues, including fibrous tissues, adipose (fat) tissues, muscle, blood vessels, bone, and cartilage such as osteogenic sarcoma, liposarcoma, fibrosarcoma, and synovial sarcoma.

Carcinosarcoma is cancer that develops in both epithelial and connective tissue. Cancer disease within the scope of this definition may be primary or secondary, whereby primary indicates that the cancer originated in the tissue where it is found rather than was established as a secondary site through metastasis from another lesion. Cancers and tumor diseases within the scope of this definition may be benign

or malign and may affect all anatomical structures of the body of a mammal. By example, to they comprise cancers and tumor diseases of I) the bone marrow and bone marrow derived cells (leukemias), II) the endocrine and exocrine glands, such as the thyroid, parathyroid, pituitary, adrenal glands, salivary glands, and pancreas III) the breast, such as benign or malignant tumors in the mammary glands of either a male or a female, the mammary ducts, adenocarcinoma, medullary carcinoma, comedocarcinoma, Paget's disease of the nipple, inflammatory carcinoma of the young woman, IV) the lung, V) the stomach, VI) the liver and spleen, VII) the small intestine, VIII) the colon, IX) the bone and its supportive and connective tissues such as malignant or benign bone tumour, such as malignant osteogenic sarcoma, benign osteoma, cartilage tumors, malignant chondrosarcoma or benign chondroma,; bone marrow tumors such as malignant myeloma or benign eosinophilic granuloma, as well as metastatic tumors from bone tissues at other locations of the body; X) the mouth, throat, larynx, and the esophagus, XI) the urinary bladder and the internal and external organs and structures of the urogenital system of male and female such as the ovaries, uterus, cervix of the uterus, testes, and prostate gland, XII) the prostate, XIII) the pancreas, such as ductal carcinoma of the pancreas; XIV) the lymphatic tissue such as lymphomas and other tumors of lymphoid origin, XV) the skin, XVI) cancers and tumor diseases of all anatomical structures belonging to the respiratory systems including thoracal muscles and linings, XVII) primary or secondary cancer of the lymph nodes, XVIII) the tongue and of the bony structures of the hard palate or sinuses, XVIV) the mouth, cheeks, neck and salivary glands, XX) the blood vessels including the heart and their linings, XXI) the smooth or skeletal muscles and their ligaments and linings, XXII) the peripheral, the autonomous, the central nervous system including the cerebellum, and XXIII) the adipose tissue.

Most standard cancer therapies target cellular proliferation and rely on the differential proliferative capacities between transformed and normal cells for their efficacy. This approach is hindered by the facts that several important normal cell types are also highly proliferative and that cancer cells frequently become resistant to

these agents. Thus, the therapeutic indices for traditional anti-cancer therapies rarely exceed 2.0.

The advent of genomics-driven molecular target identification has opened up the possibility of identifying new cancer-specific targets for therapeutic intervention that will provide safer, more effective treatments for cancer patients. Thus, newly discovered tumor-associated genes and their products can be tested for their role(s) in disease and used as tools to discover and develop innovative therapies. Genes playing important roles in any of the physiological processes outlined above can be characterized as cancer targets.

Genes or gene fragments identified through genomics can readily be expressed in one or more heterologous expression systems to produce functional recombinant proteins. These proteins are characterized *in vitro* for their biochemical properties and then used as tools in high-throughput molecular screening programs to identify chemical modulators of their biochemical activities. Agonists and/or antagonists of target protein activity can be identified in this manner and subsequently tested in cellular and *in vivo* disease models for anti-cancer activity. Optimization of lead compounds with iterative testing in biological models and detailed pharmacokinetic and toxicological analyses form the basis for drug development and subsequent testing in humans.

The esterase of the invention has similarity to phospholipases and carboxylesterases. Phospholipases are linked to cancer because of their ability to affect signal transduction by released fatty acids (PLA 1 and 2), diglyceride and ceramide (PLCs, including sphingomyelinases), or phosphatidic acid (PLDs). Additionally, a group of lipid phosphatases and transferases has been associated with cancer through their effects on the substrates for these phospholipases.

Dermatological disorders

The human esterase of the invention is highly expressed in skin. This expression demonstrates that the esterase protein or mRNA can be used to diagnose dermatological diseases. In addition, the activity of the esterase protein can be modulated to treat those diseases.

The skin serves several functions. It is a multi-layered organ system that builds an effective protective cover and regulates body temperature, senses painful and pleasant stimuli, keeps substances from entering the body, and provides a shield from the sun's harmful effects. Skin color, texture, and folds help mark people as individuals. Thus, skin disorders or diseases often have important consequences for physical and mental health. Skin disorders include, but are not limited to the conditions described in the following.

Itching (pruritus) is a sensation that instinctively demands scratching, which may be caused by a skin condition or a systemic disease.

Superficial skin disorders affect the uppermost layer of the skin, the stratum corneum or the keratin layer, which it consists of many layers of flattened, dead cells and acts as a barrier to protect the underlying tissue from injury and infection. Disorders of the superficial skin layers involve the stratum corneum and deeper layers of the epidermis. Examples of superficial skin disorders are provided in the following.

Dry skin often occurs in people past middle age, severe dry skin (ichthyosis) results from an inherited scaling disease, such as ichthyosis vulgaris or epidermolytic hyperkeratosis. Ichthyosis also results from nonhereditary disorders, such as leprosy, underactive thyroid, lymphoma, AIDS, and sarcoidosis. Keratosis pilaris is a common disorder in which dead cells shed from the upper layer of skin and form plugs that fill the openings of hair follicles. A callus is an area on the stratum corneum or keratin layer, that becomes abnormally thick in response to repeated

rubbing. A corn is a pea-sized, thickened area of keratin that occurs on the feet. Psoriasis is a chronic, recurring disease recognizable by silvery scaling bumps and various-sized plaques (raised patches). An abnormally high rate of growth and turnover of skin cells causes the scaling. Pityriasis rosea is a mild disease that causes scaly, rose-colored, inflamed skin. Pityriasis rosea is possibly caused by an infectious agent, although none has been identified. Lichen planus, a recurring itchy disease, starts as a rash of small discrete bumps that then combine and become rough, scaly plaques (raised patches).

Dermatitis (eczema) is an inflammation of the upper layers of the skin, causing blisters, redness, swelling, oozing, scabbing, scaling, and usually itching. Forms of dermatitis are contact dermatitis, or chronic dermatitis of the hands and feet, *e.g.*, Pompholyx. Further examples of dermatitic disorders are atopic dermatitis, seborrheic dermatitis, nummular dermatitis, generalized exfoliative dermatitis, stasis dermatitis, or localized scratch dermatitis (lichen simplex chronicus, neuro-dermatitis).

Other skin disorders are caused by inflammation. The skin can break out in a variety of rashes, sores, and blisters. Some skin eruptions can even be life threatening. Drug rashes are side effects of medications, mainly allergic reactions to medications.

Toxic epidermal necrolysis is a life-threatening skin disease in which the top layer of the skin peels off in sheets. This condition can be caused by a reaction to a drug or by some other serious disease.

Erythema multiforme, often caused by *Herpes simplex*, is a disorder characterized by patches of red, raised skin that often look like targets and usually are distributed symmetrically over the body. Erythema nodosum is an inflammatory disorder that produces tender red bumps (nodules) under the skin, most often over the shins but occasionally on the arms and other areas. Granuloma annulare is a chronic skin condition of unknown cause in which small, firm, raised bumps form a ring with normal or slightly sunken skin in the center.

Some skin disorders are characterized as blistering diseases. Three autoimmune diseases--pemphigus, bullous pemphigoid, and dermatitis herpetiformis--are among the most serious. Pemphigus is an uncommon, sometimes fatal, disease in which blisters (bullae) of varying sizes break out on the skin, the lining of the mouth, and other mucous membranes. Bullous pemphigoid is an autoimmune disease that causes blistering. Dermatitis herpetiform is an autoimmune disease in which clusters of intensely itchy, small blisters and hive-like swellings break out and persist. In people with the disease, proteins in wheat, rye, barley, and oat products activate the immune system, which attacks parts of the skin and somehow causes the rash and itching.

Sweating disorders also belong to skin disorders. Prickly heat is an itchy skin rash caused by trapped sweat. Excessive sweating (hyperhidrosis) may affect the entire surface of the skin, but often it is limited to the palms, soles, armpits, or groin. The affected area is often pink or bluish white, and in severe cases the skin may be cracked, scaly, and soft, especially on the feet. Skin disorders can affect the sebaceous glands. The sebaceous glands, which secrete oil onto the skin, lie in the dermis, the skin layer just below the surface layer (epidermis). Sebaceous gland disorders include acne, rosacea, perioral dermatitis, and sebaceous cysts.

Acne is a common skin condition in which the skin pores become clogged, leading to pimples and inflamed, infected abscesses (collections of pus). Acne tends to develop in teenagers. Acne is further subdivided in superficial acne or deep acne. Rosacea is a persistent skin disorder that produces redness, tiny pimples, and broken blood vessels, usually on the central area of the face. Perioral dermatitis is a red, often bumpy rash around the mouth and on the chin. A sebaceous cyst (keratinous cyst) is a slow-growing bump containing dead skin, skin excretions, and other skin particles. These cysts may be small and can appear anywhere.

Hair disorders also are skin disorders. Hair disorders include excessive hairiness, baldness, and ingrown beard hairs.

The skin can be infected by bacteria. Bacterial skin infections can range in seriousness from minor acne to a life-threatening condition, such as staphylococcal scalded skin syndrome. The most common bacterial skin infections are caused by *Staphylococcus* and *Streptococcus*. Risk factors for skin infections are for example diabetes, AIDS or skin lesions. Impetigo is a skin infection, caused by *Staphylococcus* or *Streptococcus*, leading to the formation of small pus-filled blisters (pustules). Folliculitis is an inflammation of the hair follicles caused by infection with *Staphylococcus*. The infection damages the hairs, which can be easily pulled out. Boils (furuncles) are large, tender, swollen, raised areas caused by staphylococcal infection around hair follicles. Carbuncles are clusters of boils that result in extensive sloughing of skin and scar formation. Carbuncles develop and heal more slowly than single boils and may lead to fever and fatigue.

Erysipelas is a skin infection caused by *Streptococcus*. A shiny, red, slightly swollen, tender rash develops, often with small blisters. Lymph nodes around the infected area may become enlarged and painful. Cellulitis is a spreading infection in, and sometimes beneath, the deep layers of the skin. Cellulitis most often results from a streptococcal infection or a staphylococcal infection. However, many other bacteria can also cause cellulitis. Paronychia is an infection around the edge of a fingernail or toenail. Paronychia can be caused by many different bacteria, including *Pseudomonas* and *Proteus*, and by fungi, such as *Candida*.

Staphylococcal scalded skin syndrome is a widespread skin infection that can lead to toxic shock syndrome, in which the skin peels off as though burned. Certain types of staphylococci produce a toxic substance that causes the top layer of skin (epidermis) to split from the rest of the skin. Erythrasma is an infection of the top layers of the skin by the bacterium *Corynebacterium minutissimum*.

Skin infections are often caused by fungi. Fungi that infect the skin (dermatophytes) live only in the dead, topmost layer (stratum corneum) and do not penetrate deeper.

Some fungal infections cause no symptoms or produce only a small amount of irritation, scaling, and redness. Other fungal infections cause itching, swelling, blisters, and severe scaling. Ringworm is a fungal skin infection caused by several different fungi and generally classified by its location on the body. Examples are Athlete's foot (foot ringworm, caused by either *Trichophyton* or *Epidermophyton*), jock itch (groin ringworm, can be caused by a variety of fungi and yeasts), scalp ringworm, caused by *Trichophyton* or *Microsporum*), nail ringworm and body ringworm (caused by *Trichophyton*).

Candidiasis (yeast infection, moniliasis) is an infection by the yeast *Candida*. *Candida* usually infects the skin and mucous membranes, such as the lining of the mouth and vagina. Rarely, it invades deeper tissues as well as the blood, causing life-threatening systemic candidiasis. The following types of *Candida* infections can be distinguished: Infections in skinfolds (intertriginous infections), vaginal and penile candida infections (vulvovaginitis), thrush, Perlèche (candida infection at the corners of the mouth), candidal paronychia (candida growing in the nail beds, produces painful swelling and pus). Tinea versicolor is a fungal infection that causes white to light brown patches on the skin.

The skin can also be affected by parasites, mainly tiny insects or worms. Scabies is a mite infestation that produces tiny reddish pimples and severe itching. Scabies is caused by the itch mite *Sarcoptes scabiei*. Lice infestation (pediculosis) causes intense itching and can affect almost any area of the skin. Head lice and pubic lice are two different species.

Creeping eruption (cutaneous larva migrans) is a hookworm infection transmitted from warm, moist soil to exposed skin. The infection is caused by a hookworm that normally inhabits dogs and cats.

Many types of viruses invade the skin. The medically important once cause warts and cold sores (fever blisters) on the lip. Warts are caused by the papillomavirus, and

cold sores are caused by the *Herpes simplex* virus. Another important group of viruses that infect the skin belongs to the poxvirus family. Chickenpox remains a common childhood infection. A poxvirus also causes molluscum contagiosum, which is an infection of the skin by a poxvirus that causes skin-colored, smooth, waxy bumps.

Sunlight can cause severe skin damage. Sunburn results from an overexposure to ultraviolet B (UVB) rays. Some sunburned people develop a fever, chills, and weakness, and those with very bad sunburns even may go into shock--low blood pressure, and fainting. People who are in the sun a lot have an increased risk of skin cancers, including squamous cell carcinoma, basal cell carcinoma, and to some degree, malignant melanoma. Drugs, among other causes, can cause skin photosensitivity reactions which can occur after only a few minutes of sun exposure. These reactions include redness, peeling, hives, blisters, and thickened, scaling patches (photosensitivity).

Some skin disorders are characterized as pigment disorders. Albinism is a rare, inherited disorder in which no melanin is formed. Vitiligo is a condition in which a loss of melanocytes results in smooth, whitish patches of skin, which may occur after unusual physical trauma and tends to occur with certain other diseases, including Addison's disease, diabetes, pernicious anemia, and thyroid disease. Tinea versicolor is a fungal infection of the skin that sometimes results in hyperpigmentation. Melasma appears on the face (usually the forehead, cheeks, temples, and jaws) as a roughly symmetric group of dark brown patches of pigmentation that are often clearly delineated.

Skin growths, which are abnormal accumulations of different types of cells, may be present at birth or develop later. Noncancerous (benign) growth and cancerous (malignant) growth types are distinguished. Moles (nevi) are small, usually dark, skin growths that develop from pigment-producing cells in the skin (melanocytes). Most moles are harmless. However, noncancerous moles can develop into malignant

melanoma. Skin tags are soft, small, flesh-colored or slightly darker skin flaps that appear mostly on the neck, in the armpits, or in the groin. Lipomas are soft deposits of fatty material that grow under the skin, causing round or oval lumps. Angiomas are collections of abnormally dense blood or lymph vessels that are usually located in and below the skin and that cause red or purple discolorations. Examples of angiomas are port-wine stains, strawberry marks, cavernous hemangiomas, spider angiomas, and lymphangiomas.

Pyogenic granulomas are scarlet, brown, or blue-black slightly raised areas caused by increased growth of capillaries (the smallest blood vessels) and swelling of the surrounding tissue. Seborrheic keratoses (sometimes called seborrheic warts) are flesh-colored, brown, or black growths that can appear anywhere on the skin. Dermatofibromas are small, red-to-brown bumps (nodules) that result from an accumulation of fibroblasts, the cells that populate the soft tissue under the skin.

Keratoacanthomas are round, firm, usually flesh-colored growths that have an unusual central crater containing a pasty material. Keloids are smooth, shiny, slightly pink, often dome-shaped, proliferative growths of fibrous tissue that form over areas of injury or over surgical wounds.

Skin cancer is the most common form of cancer, but most types of skin cancers are curable. Basal cell carcinoma is a cancer that originates in the lowest layer of the epidermis. Squamous cell carcinoma is cancer that originates in the middle layer of the epidermis. Bowen's disease is a form of squamous cell carcinoma that's confined to the epidermis and hasn't yet invaded the underlying dermis. Melanoma is a cancer that originates in the pigment-producing cells of the skin (melanocytes). Kaposi's sarcoma is a cancer that originates in the blood vessels, usually of the skin. Paget's disease is a rare type of skin cancer that looks like an inflamed, reddened patch of skin (dermatitis); it originates in glands in or under the skin.

Gastrointestinal and liver disorders

The human esterase of the invention is highly expressed in esophagus, esophageal tumor, stomach tumor, colon, colon tumor, small intestine, ileum, ileum tumor, rectum, liver, cirrhotic liver, liver tumor, and HEP G2 cells. The expression in the above-mentioned tissues and in particular the differential expression between diseased tissue and healthy tissue, demonstrates that the human esterase protein or mRNA can be used to diagnose gastroenterological disorders. In addition, the activity of the protein can be modulated to treat gastroenterological disorders.

Gastrointestinal diseases include primary or secondary, acute or chronic diseases of the organs of the gastrointestinal tract which may be acquired or inherited, benign or malignant or metaplastic, and which may affect the organs of the gastrointestinal tract or the body as a whole. They include but are not limited to 1) disorders of the esophagus such as achalasia, vigoruos achalasia, dysphagia, cricopharyngeal incoordination, pre-esophageal dysphagia, diffuse esophageal spasm, globus sensation, Barrett's metaplasia, gastroesophageal reflux, 2) disorders of the stomach and duodenum such as functional dyspepsia, inflammation of the gastric mucosa, gastritis, stress gastritis, chronic erosive gastritis, atrophy of gastric glands, metaplasia of gastric tissues, gastric ulcers, duodenal ulcers, neoplasms of the stomach, 3) disorders of the pancreas such as acute or chronic pancreatitis, insufficiency of the exocrinic or endocrinic tissues of the pancreas such as steatorrhea, diabetes, neoplasms of the exocrine or endocrine pancreas such as 3.1) multiple endocrine neoplasia syndrome, ductal adenocarcinoma, cystadeno-carcinoma, islet cell tumors, insulinoma, gastrinoma, carcinoid tumors, glucagonoma, Zollinger-Ellison syndrome, Vipoma syndrome, malabsorption syndrome, 4) disorders of the bowel such as chronic inflammatory diseases of the bowel, Crohn's disease, ileus, diarrhea and constipation, colonic inertia, megacolon, malabsorption syndrome, ulcerative colitis, 4.1) functional bowel disorders such as irritable bowel syndrome, 4.2) neoplasms of the bowel such as familial polyposis,

adenocarcinoma, primary malignant lymphoma , carcinoid tumors, Kaposi's sarcoma, polyps, cancer of the colon and rectum.

Liver diseases include primary or secondary, acute or chronic diseases or injury of the liver which may be acquired or inherited, benign or malignant, and which may affect the liver or the body as a whole. They comprise but are not limited to disorders of the bilirubin metabolism, jaundice, syndromes of Gilbert, Crigler-Najjar, Dubin-Johnson, and Rotor; intrahepatic cholestasis, hepatomegaly, portal hypertension, ascites, Budd-Chiari syndrome, portal-systemic encephalopathy, fatty liver, steatosis, Reye's syndrome, liver diseases due to alcohol, alcoholic hepatitis or cirrhosis, fibrosis and cirrhosis, fibrosis and cirrhosis of the liver due to inborn errors of metabolism or exogenous substances, storage diseases, syndromes of Gaucher and Zellweger, Wilson's disease, acute or chronic hepatitis, viral hepatitis and its variants, inflammatory conditions of the liver due to viruses, bacteria , fungi, protozoa, helminths; drug induced disorders of the liver, chronic liver diseases such as primary sclerosing cholangitis, alpha1-antitrypsin-deficiency, primary biliary cirrhosis, postoperative liver disorders such as postoperative intrahepatic cholestasis, hepatic granulomas, vascular liver disorders associated with systemic disease, benign or malignant neoplasms of the liver, disturbance of liver metabolism in the new-born or prematurely born.

Metabolic disorders

The human esterase protein of the invention is highly expressed in liver and cirrhotic liver. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue and healthy tissue demonstrates that esterase protein or mRNA can be used to diagnose metabolic diseases. In addition, the activity of the esterase protein can be modulated to treat metabolic diseases.

Metabolic disorders are defined as conditions that result from an abnormality in any of the chemical or biochemical transformations and their regulating systems essential

to producing energy, to regenerating cellular constituents, to eliminating unneeded products arising from these processes, and to regulate and maintain homeostasis in a mammal regardless of whether acquired or the result of a genetic transformation. Depending on which metabolic pathway is involved, a single defective transformation or disturbance of its regulation may produce consequences that are narrow, involving a single body function, or broad, affecting many organs, organ systems, or the body as a whole. Diseases resulting from abnormalities related to the fine and coarse mechanisms that affect each individual transformation, its rate and direction, or the availability of substrates like amino acids, fatty acids, carbohydrates, minerals, cofactors, hormones, regardless whether they are inborn or acquired, are well within the scope of the definition of a metabolic disease according to this application.

Metabolic disorders often are caused by single defects in particular biochemical pathways, defects that are due to the deficient activity of individual enzymes or molecular receptors leading to the regulation of such enzymes. Hence, in a broader sense disturbances of the underlying genes, their products and their regulation lie well within the scope of this definition of a metabolic disease. For example, metabolic disorders may affect 1) biochemical processes and tissues ubiquitous all over the body, 2) the bone, 3) the nervous system, 4) the endocrine system, 5) the muscle including the heart, 6) the skin and nervous tissue, 7) the urogenital system, 8) the homeostasis of body systems like water and electrolytes.

Metabolic disorders according to 1) include, but are not limited to, obesity, amyloidosis, disturbances of the amino acid metabolism like branched chain disease, hyperaminoacidemia, hyperaminoaciduria, disturbances of the metabolism of urea, hyperammonemia, mucopolysaccharidoses (*e.g.*, Maroteaux-Lamy syndrome, storage diseases such as glycogen storage diseases and lipid storage diseases, glycogenosis diseases such as Cori's disease, malabsorption diseases such as intestinal carbohydrate malabsorption, oligosaccharidase deficiency like maltase-, lactase-, or sucrase-insufficiency, disorders of the metabolism of fructose, disorders of the metabolism of galactose, galactosemia, disturbances of carbohydrate

utilization such as diabetes, hypoglycemia, disturbances of pyruvate metabolism, hypolipidemia, hypolipoproteinemia, hyperlipidemia, hyperlipoproteinemia, carnitine or carnitine acyltransferase deficiency, disturbances of the porphyrin metabolism, porphyrias, disturbances of the purine metabolism, lysosomal diseases, and metabolic disorders of nerves and nervous systems such as gangliosidoses, sphingolipidoses, sulfatidoses, leucodystrophies, and Lesch-Nyhan syndrome.

Metabolic disorders according to 2) include, but are not limited to, osteoporosis, osteomalacia-like osteoporosis, osteopenia, osteogenesis imperfecta, osteopetrosis, osteonecrosis, Paget's disease of bone, and hypophosphatemia.

Metabolic disorders according to 3) include, but are not limited to, cerebellar dysfunction, disturbances of brain metabolism such as dementia, Alzheimer's disease, Huntington's chorea, Parkinson's disease, Pick's disease, toxic encephalopathy, demyelinating neuropathies such as inflammatory neuropathy, and Guillain-Barré syndrome.

Metabolic disorders according to 4) include, but are not limited to, primary and secondary metabolic disorders associated with hormonal defects such as any disorder stemming from either a hyperfunction or hypofunction of some hormone-secreting endocrine gland and any combination thereof. They include Sipple's syndrome, pituitary gland dysfunction and its effects on other endocrine glands, such as the thyroid, adrenals, ovaries, and testes, acromegaly, hyper- and hypothyroidism, euthyroid goiter, euthyroid sick syndrome, thyroiditis, and thyroid cancer, over- or underproduction of the adrenal steroid hormones, adrenogenital syndrome, Cushing's syndrome, Addison's disease of the adrenal cortex, Addison's pernicious anemia, primary and secondary aldosteronism, diabetes insipidus, carcinoid syndrome, disturbances caused by the dysfunction of the parathyroid glands, pancreatic islet cell dysfunction, diabetes, disturbances of the endocrine system of the female such as estrogen deficiency, and resistant ovary syndrome.

Metabolic disorders according to 5) include, but are not limited to, muscle weakness, myotonia, Duchenne's and other muscular dystrophies, dystrophia myotonica of Steinert, mitochondrial myopathies such as disturbances of the catabolic metabolism in the muscle, carbohydrate and lipid storage myopathies, glycogenoses, myoglobinuria, malignant hyperthermia, polymyalgia rheumatica, dermatomyositis, primary myocardial disease, cardiomyopathy.

Metabolic disorders according to 6) include, but are not limited to, disorders of the ectoderm, neurofibromatosis, scleroderma and polyarteritis, Louis-Bar syndrome, von Hippel-Lindau disease, Sturge-Weber syndrome, tuberous sclerosis, amyloidosis, porphyria.

Metabolic disorders according to 7) include, but are not limited to, sexual dysfunction of the male and female.

Metabolic disorders according to 8) include, but are not limited to, confused states and seizures due to inappropriate secretion of antidiuretic hormone from the pituitary gland, Liddle's syndrome, Bartter's syndrome, Fanconi's syndrome, renal electrolyte wasting, diabetes insipidus.

Inflammatory disorders

The human esterase of the invention is highly expressed in the following tissues of the immune system and tissues responsive to components of the immune system as well as in the following tissues responsive to mediators of inflammation: liver and cirrhotic liver. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue and healthy tissue demonstrates that the esterase protein or mRNA can be used to diagnose inflammatory diseases. In addition, the activity of the esterase protein can be modulated to treat inflammatory diseases.

Inflammatory diseases include diseases triggered by cellular or non-cellular mediators of the immune system or tissues causing the inflammation of body tissues and subsequently producing an acute or chronic inflammatory condition. Examples of such inflammatory diseases are hypersensitivity reactions of type I — IV, *e.g.*, hypersensitivity diseases of the lung including asthma, atopic diseases, allergic rhinitis or conjunctivitis, angioedema of the lids, hereditary angioedema, antireceptor hypersensitivity reactions and autoimmune diseases, Hashimoto's thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, pemphigus, myasthenia gravis, Grave's and Raynaud's disease, type B insulin-resistant diabetes, rheumatoid arthritis, psoriasis, Crohn's disease, scleroderma, mixed connective tissue disease, polymyositis, sarcoidosis, glomerulonephritis, acute or chronic host versus graft reactions.

Reproductive disorders

The esterase protein of the invention is highly expressed in the following tissues of the reproductive system: placenta, uterus tumor, ovary, ovary tumor, breast, breast tumor, MDA MB 231 cells (breast tumor), mammary gland. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue and healthy tissue demonstrates that the protein or mRNA can be used to diagnose reproduction disorders. In addition, the activity of the esterase protein can be modulated to treat reproductive disorders.

Disorders of the male reproductive system include but are not limited to balanoposthitis, balanitis xerotica obliterans, phimosis, paraphimosis, erythroplasia of Queyrat, skin cancer of the penis, Bowen's and Paget's diseases, syphilis, herpes simplex infections, genital warts, molluscum contagiosum, priapism, peyronie's disease, benign prostatic hyperplasia (BPH), prostate cancer, prostatitis, testicular cancer, testicular torsion, inguinal hernia, epididymo-orchitis, mumps, hydroceles, spermatoceles, or varicoceles.

Impotence (erectile dysfunction) may results from vascular impairment, neurologic disorders, drugs, abnormalities of the penis, or psychological problems.

Examples of disorders of the female reproductive include premature menopause, pelvic pain, vaginitis, vulvitis, vulvovaginitis, pelvic inflammatory disease, fibroids, menstrual disorders (premenstrual syndrome (PMS), dysmenorrhea, amenorrhea, primary amenorrhea, secondary amenorrhea, menorrhagia, hypomenorrhea, polymenorrhea, oligomenorrhea, metrorrhagia, menometrorrhagia, Postmenopausal bleeding), bleeding caused by a physical disorder, dysfunctional uterine bleeding, polycystic ovary syndrome (Stein-Leventhal syndrome), endometriosis, cancer of the uterus, cancer of the cervix, cancer of the ovaries, cancer of the vulva, cancer of the vagina, cancer of the fallopian tubes, and hydatidiform mole.

Infertility may be caused by problems with sperm, ovulation, the fallopian tubes, and the cervix as well as unidentified factors.

Complications of pregnancy include miscarriage and stillbirth, ectopic pregnancy, anemia, Rh incompatibility, problems with the placenta, excessive vomiting, preeclampsia, eclampsia, and skin rashes (e.g. herpes gestationis, urticaria of pregnancy) as well as preterm labor and premature rupture of the membranes.

Breast disorders may be noncancerous (benign) or cancerous (malignant). Examples of breast disorders are but are not limited to breast pain, cysts, fibrocystic breast disease, fibrous lumps, nipple discharge, breast infection, breast cancer (ductal carcinoma, lobular carcinoma, medullary carcinoma, tubular carcinoma, and inflammatory breast cancer), Paget's disease of the nipple or Cystosarcoma phyllodes.

Genitourinary disorders

The human esterase protein of the invention is highly expressed in the following urological tissues: prostate, prostate BPH, ureter, fetal kidney, kidney, HEK 293

cells. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue and healthy tissue demonstrates that the esterase protein or mRNA can be used to diagnose urological disorders. In addition, the activity of the esterase protein can be modulated to treat urological disorders.

Genitourinary disorders include benign and malign disorders of the organs constituting the genitourinary system of female and male, renal diseases such as acute or chronic renal failure, immunologically mediated renal diseases such as renal transplant rejection, lupus nephritis, immune complex renal diseases, glomerulopathies, nephritis, toxic nephropathy, obstructive uropathies such as benign prostatic hyperplasia (BPH), neurogenic bladder syndrome, urinary incontinence such as urge-, stress-, or overflow incontinence, pelvic pain, and erectile dysfunction.

Neurological disorders

The human esterase protein of the invention is highly expressed in the following brain tissues: fetal brain, brain, Alzheimer brain, cerebellum (right), cerebellum (left), cerebral cortex, Alzheimer cerebral cortex, frontal lobe, Alzheimer brain frontal lobe, occipital lobe, parietal lobe, temporal lobe, precentral gyrus, tonsilla cerebelli, vermis cerebelli, pons, substantia nigra, cerebral peduncles, corpus callosum, hippocampus, spinal cord, neuroblastoma SH-SY5Y cells, neuroblastoma IMR32 cells, glial tumor H4 cells, HEK CNS. The expression in brain tissues and in particular the differential expression between diseased tissue and healthy tissue demonstrates that the esterase protein or mRNA can be used to diagnose nervous system diseases. In addition, the activity of the esterase protein can be modulated to treat nervous system diseases.

Neurological disorders include disorders of the central nervous system as well as disorders of the peripheral nervous system. CNS disorders include, but are not limited to, brain injuries, cerebrovascular diseases and their consequences, Parkinson's disease, corticobasal degeneration, motor neuron disease (including

ALS), multiple sclerosis, traumatic brain injury, stroke, post-stroke, post-traumatic brain injury, and small-vessel cerebrovascular disease. Dementias, such as Alzheimer's disease, vascular dementia, dementia with Lewy bodies, frontotemporal dementia and Parkinsonism linked to chromosome 17, frontotemporal dementias (including Pick's disease), progressive nuclear palsy, corticobasal degeneration, Huntington's disease, thalamic degeneration, Creutzfeld-Jakob dementia, HIV dementia, schizophrenia with dementia, and Korsakoff's psychosis, also are CNS disorders.

Similarly, cognitive-related disorders, such as mild cognitive impairment, age-associated memory impairment, age-related cognitive decline, vascular cognitive impairment, attention deficit disorders, attention deficit hyperactivity disorders, and memory disturbances in children with learning disabilities also are considered to be CNS disorders.

Pain, within the meaning of the invention, is also considered to be a CNS disorder. Pain can be associated with CNS disorders, such as multiple sclerosis, spinal cord injury, sciatica, failed back surgery syndrome, traumatic brain injury, epilepsy, Parkinson's disease, post-stroke, and vascular lesions in the brain and spinal cord (*e.g.*, infarct, hemorrhage, vascular malformation). Non-central neuropathic pain includes that associated with post mastectomy pain, phantom feeling, reflex sympathetic dystrophy (RSD), trigeminal neuralgiaradioculopathy, post-surgical pain, HIV/AIDS related pain, cancer pain, metabolic neuropathies (*e.g.*, diabetic neuropathy, vasculitic neuropathy secondary to connective tissue disease), paraneoplastic polyneuropathy associated, for example, with carcinoma of lung, or leukemia, or lymphoma, or carcinoma of prostate, colon or stomach, trigeminal neuralgia, cranial neuralgias, and post-herpetic neuralgia. Pain is also associated with peripheral nerve damage, central pain (*e.g.*, due to cerebral ischemia) and various chronic pain (*e.g.*, lumbago, back pain (low back pain), inflammatory and/or rheumatic pain. Headache pain (for example, migraine with aura, migraine without aura, and other migraine disorders), episodic and chronic tension-type headache,

tension-type like headache, cluster headache, and chronic paroxysmal hemicrania also are CNS disorders. Visceral pain, such as pancreatits, intestinal cystitis, dysmenorrhea, irritable Bowel syndrome, Crohn's disease, biliary colic, ureteral colic, myocardial infarction and pain syndromes of the pelvic cavity, *e.g.*, vulvodynia, orchialgia, urethral syndrome and protatodynia also is a CNS disorder. Also considered to be disorders of the nervous system are acute pain, for example postoperative pain, and pain after trauma.

Respiratory disorders

The human esterase of the invention is highly expressed in the following tissues of the respiratory system: fetal lung, fetal lung fibroblast IMR-90 cells, fetal lung fibroblast MRC-5 cells, lung, lung right lower lobe, lung tumor, trachea. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue and healthy tissue demonstrates that the esterase protein or mRNA can be used to diagnose respiratory diseases. In addition, the activity of the esterase protein can be modulated to treat those diseases.

Asthma

Allergy is a complex process in which environmental antigens induce clinically adverse reactions. The inducing antigens, called allergens, typically elicit a specific IgE response and, although in most cases the allergens themselves have little or no intrinsic toxicity, they induce pathology when the IgE response in turn elicits an IgE-dependent or T cell-dependent hypersensitivity reaction. Hypersensitivity reactions can be local or systemic and typically occur within minutes of allergen exposure in individuals who have previously been sensitized to an allergen. The hypersensitivity reaction of allergy develops when the allergen is recognized by IgE antibodies bound to specific receptors on the surface of effector cells, such as mast cells, basophils, or eosinophils, which causes the activation of the effector cells and the release of mediators that produce the acute signs and symptoms of the reactions.

Allergic diseases include asthma, allergic rhinitis (hay fever), atopic dermatitis, and anaphylaxis.

Asthma is thought to arise as a result of interactions between multiple genetic and environmental factors and is characterized by three major features: 1) intermittent and reversible airway obstruction caused by bronchoconstriction, increased mucus production, and thickening of the walls of the airways that leads to a narrowing of the airways, 2) airway hyperresponsiveness caused by a decreased control of airway caliber, and 3) airway inflammation. Certain cells are critical to the inflammatory reaction of asthma and they include T cells and antigen presenting cells, B cells that produce IgE, and mast cells, basophils, eosinophils, and other cells that bind IgE. These effector cells accumulate at the site of allergic reaction in the airways and release toxic products that contribute to the acute pathology and eventually to the tissue destruction related to the disorder. Other resident cells, such as smooth muscle cells, lung epithelial cells, mucus-producing cells, and nerve cells may also be abnormal in individuals with asthma and may contribute to the pathology. While the airway obstruction of asthma, presenting clinically as an intermittent wheeze and shortness of breath, is generally the most pressing symptom of the disease requiring immediate treatment, the inflammation and tissue destruction associated with the disease can lead to irreversible changes that eventually make asthma a chronic disabling disorder requiring long-term management.

Despite recent important advances in our understanding of the pathophysiology of asthma, the disease appears to be increasing in prevalence and severity (Gergen and Weiss, *Am. Rev. Respir. Dis. 146*, 823-24, 1992). It is estimated that 30-40% of the population suffer with atopic allergy, and 15% of children and 5% of adults in the population suffer from asthma (Gergen and Weiss, 1992). Thus, an enormous burden is placed on our health care resources. However, both diagnosis and treatment of asthma are difficult. The severity of lung tissue inflammation is not easy to measure and the symptoms of the disease are often indistinguishable from those of respiratory infections, chronic respiratory inflammatory disorders, allergic rhinitis, or other

respiratory disorders. Often, the inciting allergen cannot be determined, making removal of the causative environmental agent difficult. Current pharmacological treatments suffer their own set of disadvantages. Commonly used therapeutic agents, such as beta agonists, can act as symptom relievers to transiently improve pulmonary function, but do not affect the underlying inflammation. Agents that can reduce the underlying inflammation, such as anti-inflammatory steroids, can have major drawbacks that range from immunosuppression to bone loss (Goodman and Gilman's THE PHARMACOLOGIC BASIS OF THERAPEUTICS, Seventh Edition, MacMillan Publishing Company, NY, USA, 1985). In addition, many of the present therapies, such as inhaled corticosteroids, are short-lasting, inconvenient to use, and must be used often on a regular basis, in some cases for life, making failure of patients to comply with the treatment a major problem and thereby reducing their effectiveness as a treatment.

Because of the problems associated with conventional therapies, alternative treatment strategies have been evaluated. Glycophorin A (Chu and Sharom, *Cell. Immunol. 145*, 223-39, 1992), cyclosporin (Alexander *et al.*, *Lancet 339*, 324-28, 1992), and a nonapeptide fragment of IL-2 (Zav'yalov *et al.*, *Immunol. Lett. 31*, 285-88, 1992) all inhibit interleukin-2 dependent T lymphocyte proliferation; however, they are known to have many other effects. For example, cyclosporin is used as a immuno-suppressant after organ transplantation. While these agents may represent alternatives to steroids in the treatment of asthmatics, they inhibit interleukin-2 dependent T lymphocyte proliferation and potentially critical immune functions associated with homeostasis. Other treatments that block the release or activity of mediators of bronchochonstriction, such as cromones or anti-leukotrienes, have recently been introduced for the treatment of mild asthma, but they are expensive and not effective in all patients and it is unclear whether they have any effect on the chronic changes associated with asthmatic inflammation. What is needed in the art is the identification of a treatment that can act in pathways critical to the development of asthma that both blocks the episodic attacks of the disorder and preferentially

dampens the hyperactive allergic immune response without immunocompromising the patient.

COPD

Chronic obstructive pulmonary (or airways) disease (COPD) is a condition defined physiologically as airflow obstruction that generally results from a mixture of emphysema and peripheral airway obstruction due to chronic bronchitis (Senior & Shapiro, *Pulmonary Diseases and Disorders*, 3d ed., New York, McGraw-Hill, 1998, pp. 659-681, 1998; Barnes, *Chest 117*, 10S-14S, 2000). Emphysema is characterized by destruction of alveolar walls leading to abnormal enlargement of the air spaces of the lung. Chronic bronchitis is defined clinically as the presence of chronic productive cough for three months in each of two successive years. In COPD, airflow obstruction is usually progressive and is only partially reversible. By far the most important risk factor for development of COPD is cigarette smoking, although the disease does occur in non-smokers.

Chronic inflammation of the airways is a key pathological feature of COPD (Senior & Shapiro, 1998). The inflammatory cell population comprises increased numbers of macrophages, neutrophils, and $CD8^+$ lymphocytes. Inhaled irritants, such as cigarette smoke, activate macrophages that are resident in the respiratory tract, as well as epithelial cells leading to release of chemokines (*e.g.*, interleukin-8) and other chemotactic factors. These chemotactic factors act to increase the neutrophil/-monocyte trafficking from the blood into the lung tissue and airways. Neutrophils and monocytes recruited into the airways can release a variety of potentially damaging mediators such as proteolytic enzymes and reactive oxygen species. Matrix degradation and emphysema, along with airway wall thickening, surfactant dysfunction, and mucus hypersecretion, all are potential sequelae of this inflammatory response that lead to impaired airflow and gas exchange.

This invention further pertains to the use of novel agents identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a human esterase polypeptide binding molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

A reagent which affects esterase activity can be administered to a human cell, either *in vitro* or *in vivo*, to reduce esterase activity. The reagent preferably binds to an expression product of a human esterase gene. If the expression product is a protein, the reagent is preferably an antibody. For treatment of human cells *ex vivo*, an antibody can be added to a preparation of stem cells that have been removed from the body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

In one embodiment, the reagent is delivered using a liposome. Preferably, the liposome is stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal, such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its

contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5 μg of DNA per 16 nmole of liposome delivered to about 10^6 cells, more preferably about 1.0 μg of DNA per 16 nmole of liposome delivered to about 10^6 cells, and even more preferably about 2.0 μg of DNA per 16 nmol of liposome delivered to about 10^6 cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods that are standard in the art (see, for example, U.S. Patent 5,705,151). Preferably, from about 0.1 μg to about 10 μg of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5 μg to about 5 μg of polynucleotides are combined with about 8 nmol liposomes, and even more preferably about 1.0 μg of polynucleotides is combined with about 8 nmol liposomes.

In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are taught in, for example, Findeis *et al. Trends in Biotechnol. 11*, 202-05 (1993); Chiou *et al.*, GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER (J.A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem. 263*, 621-24 (1988); Wu *et al.*, *J. Biol. Chem. 269*, 542-46 (1994); Zenke *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 87*, 3655-59 (1990); Wu *et al.*, *J. Biol. Chem. 266*, 338-42 (1991).

Determination of a therapeutically effective dose

The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which increases or decreases enzymatic activity relative to the enzymatic activity which occurs in the absence of the therapeutically effective dose.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

Therapeutic efficacy and toxicity, *e.g.*, ED_{50} (the dose therapeutically effective in 50% of the population) and LD_{50} (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD_{50}/ED_{50}.

Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors that can be taken into account include the severity of the disease state,

general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

If the reagent is a single-chain antibody, polynucleotides encoding the antibody can be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation–mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium phosphate-mediated transfection.

Effective *in vivo* dosages of an antibody are in the range of about 5 µg to about 50 µg/kg, about 50 µg to about 5 mg/kg, about 100 µg to about 500 µg/kg of patient body weight, and about 200 to about 250 µg/kg of patient body weight. For administration of polynucleotides encoding single-chain antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of DNA.

If the expression product is mRNA, the reagent is preferably an antisense oligonucleotide or a ribozyme. Polynucleotides that express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above.

Preferably, a reagent reduces expression of a human esterase gene or the activity of an esterase polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of expression of a human esterase gene or the activity of a human esterase polypeptide can be assessed using methods well known in the art, such as hybridization of nucleotide probes to esterase-specific mRNA, quantitative RT-PCR, immunologic detection of a human esterase polypeptide, or measurement of enzymatic activity.

In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

Diagnostic methods

Human esterase also can be used in diagnostic assays for detecting diseases and abnormalities or susceptibility to diseases and abnormalities related to the presence of mutations in the nucleic acid sequences that encode the enzyme. For example,

differences can be determined between the cDNA or genomic sequence encoding esterase in individuals afflicted with a disease and in normal individuals. If a mutation is observed in some or all of the afflicted individuals but not in normal individuals, then the mutation is likely to be the causative agent of the disease.

Sequence differences between a reference gene and a gene having mutations can be revealed by the direct DNA sequencing method. In addition, cloned DNA segments can be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer can be used with a double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures using radiolabeled nucleotides or by automatic sequencing procedures using fluorescent tags.

Genetic testing based on DNA sequence differences can be carried out by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized, for example, by high resolution gel electrophoresis. DNA fragments of different sequences can be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (*see, e.g.*, Myers *et al.*, *Science 230*, 1242, 1985). Sequence changes at specific locations can also be revealed by nuclease protection assays, such as RNase and S 1 protection or the chemical cleavage method (*e.g.*, Cotton *et al.*, *Proc. Natl. Acad. Sci. USA 85*, 4397-4401, 1985). Thus, the detection of a specific DNA sequence can be performed by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes and Southern blotting of genomic DNA. In addition to direct methods such as gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

Altered levels of esterase also can be detected in various tissues. Assays used to detect levels of the receptor polypeptides in a body sample, such as blood or a tissue biopsy, derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive binding assays, Western blot analysis, and ELISA assays.

All patents and patent applications cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

Detection of esterase activity

The polynucleotide of SEQ ID NO: 1 is inserted into the expression vector pCEV4 and the expression vector pCEV4-esterase polypeptide obtained is transfected into human embryonic kidney 293 cells. From these cells extracts are obtained and samples containing either 1 mM CaCl2 or 1 mM EDTA are incubated at 37°C in a shaking water bath (15 min). PA in ethanol is added to initiate the reaction (1 mM final concentration). The reaction mixtures are incubated for an additional time period (within the linear range of the reaction) and stopped with 0.5 ml of 0.4% aminoantipyrine in 5% SDS. An aliquot of 0.25 ml 1 % potassium ferricyanide is added to develop color. Triplicate subsamples are run. Absorbances are read at 510 nm and the amount of phenol produced is calculated using a molar extinction coefficient of 1.4162 x 104 M-1 cm-1. It is shown that the polypeptide of SEQ ID NO: 2 has an esterase activity.

EXAMPLE 2

Expression of recombinant human esterase

The *Pichia pastoris* expression vector pPICZB (Invitrogen, San Diego, CA) is used to produce large quantities of recombinant human esterase polypeptides in yeast. The esterase-encoding DNA sequence is derived from SEQ ID NO: 1. Before insertion into vector pPICZB, the DNA sequence is modified by well known methods in such a way that it contains at its 5'-end an initiation codon and at its 3'-end an enterokinase cleavage site, a His6 reporter tag and a termination codon. Moreover, at both termini recognition sequences for restriction endonucleases are added and after digestion of the multiple cloning site of pPICZ B with the corresponding restriction enzymes the modified DNA sequence is ligated into pPICZB. This expression vector

is designed for inducible expression in *Pichia pastoris*, driven by a yeast promoter. The resulting pPICZ/md-His6 vector is used to transform the yeast.

The yeast is cultivated under usual conditions in 5 liter shake flasks and the recombinantly produced protein isolated from the culture by affinity chromatography (Ni-NTA-Resin) in the presence of 8 M urea. The bound polypeptide is eluted with buffer, pH 3.5, and neutralized. Separation of the polypeptide from the His6 reporter tag is accomplished by site-specific proteolysis using enterokinase (Invitrogen, San Diego, CA) according to manufacturer's instructions. Purified human esterase polypeptide is obtained.

EXAMPLE 3

Identification of test compounds that bind to esterase polypeptides

Purified esterase polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well microtiter plates are contacted with test compounds from a small molecule library at pH 7.0 in a physiological buffer solution. Human esterase polypeptides comprise the amino acid sequence shown in SEQ ID NO: 2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

The buffer solution containing the test compounds is washed from the wells. Binding of a test compound to a human esterase polypeptide is detected by fluorescence measurements of the contents of the wells. A test compound that increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound which binds to a human esterase polypeptide.

EXAMPLE 4

Identification of a test compound which decreases esterase gene expression

A test compound is administered to a culture of human cells transfected with an esterase expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control.

RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem. 18*, 5294-99, 1979). Northern blots are prepared using 20 to 30 µg total RNA and hybridized with a ^{32}P-labeled esterase-specific probe at 65°C in Express-hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides selected from the complement of SEQ ID NO: 1. A test compound that decreases the esterase-specific signal relative to the signal obtained in the absence of the test compound is identified as an inhibitor of esterase gene expression.

EXAMPLE 5

Identification of a test compound which decreases esterase activity

A test compound is administered to a culture of human cells transfected with an esterase expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control. Esterase activity is measured using methods well known in the art. A test compound which decreases the enzymatic activity of the esterase relative to the enzymatic activity in the absence of the test compound is identified as an inhibitor of esterase activity.

EXAMPLE 6

Tissue-specific expression of esterase

The qualitative expression pattern of esterase in various tissues is determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Quantitative expression profiling.

To demonstrate that esterase is involved in cancer, expression is determined in the following tissues: adrenal gland, bone marrow, brain, cerebellum, colon, fetal brain, fetal liver, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spinal cord, spleen, stomach, testis, thymus, thyroid, trachea, uterus, and peripheral blood lymphocytes. Expression in the following cancer cell lines also is determined: DU-145 (prostate), NCI-H125 (lung), HT-29 (colon), COLO-205 (colon), A-549 (lung), NCI-H460 (lung), HT-116 (colon), DLD-1 (colon), MDA-MD-231 (breast), LS174T (colon), ZF-75 (breast), MDA-MN-435 (breast), HT-1080, MCF-7 (breast), and U87. Matched pairs of malignant and normal tissue from the same patient also are tested.

Quantitative expression profiling is performed by the form of quantitative PCR analysis called "kinetic analysis" firstly described in Higuchi *et al.*, *BioTechnology 10*, 413-17, 1992, and Higuchi *et al.*, *BioTechnology 11*, 1026-30, 1993. The principle is that at any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies.

If the amplification is performed in the presence of an internally quenched fluorescent oligonucleotide (TaqMan probe) complementary to the target sequence, the probe is cleaved by the 5'-3' endonuclease activity of Taq DNA polymerase and a fluorescent dye released in the medium (Holland *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 88*, 7276-80, 1991). Because the fluorescence emission will increase in direct

proportion to the amount of the specific amplified product, the exponential growth phase of PCR product can be detected and used to determine the initial template concentration (Heid *et al., Genome Res. 6*, 986-94, 1996, and Gibson *et al., Genome Res. 6*, 995-1001, 1996).

The amplification of an endogenous control can be performed to standardize the amount of sample RNA added to a reaction. In this kind of experiment, the control of choice is the 18S ribosomal RNA. Because reporter dyes with differing emission spectra are available, the target and the endogenous control can be independently quantified in the same tube if probes labeled with different dyes are used. All "real time PCR" measurements of fluorescence are made in the ABI Prism 7700.

RNA extraction and cDNA preparation. Total RNA from the tissues listed above are used for expression quantification. RNAs labeled "from autopsy" were extracted from autoptic tissues with the TRIzol reagent (Life Technologies, MD) according to the manufacturer's protocol.

Fifty µg of each RNA were treated with DNase I for 1 hour at 37°C in the following reaction mix: 0.2 U/µl RNase-free DNase I (Roche Diagnostics, Germany); 0.4 U/µl RNase inhibitor (PE Applied Biosystems, CA); 10 mM Tris-HCl pH 7.9; 10 mM $MgCl_2$; 50 mM NaCl; and 1 mM DTT.

After incubation, RNA is extracted once with 1 volume of phenol:chloroform:-isoamyl alcohol (24:24:1) and once with chloroform, and precipitated with 1/10 volume of 3 M sodium acetate, pH5.2, and 2 volumes of ethanol.

Fifty µg of each RNA from the autoptic tissues are DNase treated with the DNA-free kit purchased from Ambion (Ambion, TX). After resuspension and spectrophotometric quantification, each sample is reverse transcribed with the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA) according to the manufacturer's protocol. The final concentration of RNA in the reaction mix is

200 ng/μL. Reverse transcription is carried out with 2.5 μM of random hexamer primers.

TaqMan quantitative analysis. Specific primers and probe are designed according to the recommendations of PE Applied Biosystems; the probe can be labeled at the 5' end FAM (6-carboxy-fluorescein) and at the 3' end with TAMRA (6-carboxy-tetramethyl-rhodamine). Quantification experiments are performed on 10 ng of reverse transcribed RNA from each sample. Each determination is done in triplicate.

Total cDNA content is normalized with the simultaneous quantification (multiplex PCR) of the 18S ribosomal RNA using the Pre-Developed TaqMan Assay Reagents (PDAR) Control Kit (PE Applied Biosystems, CA).

The assay reaction mix is as follows: 1X final TaqMan Universal PCR Master Mix (from 2X stock) (PE Applied Biosystems, CA); 1X PDAR control – 18S RNA (from 20X stock); 300 nM forward primer; 900 nM reverse primer; 200 nM probe; 10 ng cDNA; and water to 25 μl.

Each of the following steps are carried out once: pre PCR, 2 minutes at 50°C, and 10 minutes at 95°C. The following steps are carried out 40 times: denaturation, 15 seconds at 95°C, annealing/extension, 1 minute at 60°C.

The experiment is performed on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems, CA). At the end of the run, fluorescence data acquired during PCR are processed as described in the ABI Prism 7700 user's manual in order to achieve better background subtraction as well as signal linearity with the starting target quantity.

EXAMPLE 7

Proliferation inhibition assay: Antisense oligonucleotides suppress the growth of cancer cell lines

The cell line used for testing is the human colon cancer cell line HCT116. Cells are cultured in RPMI-1640 with 10-15% fetal calf serum at a concentration of 10,000 cells per milliliter in a volume of 0.5 ml and kept at 37°C in a 95% air/5%CO_2 atmosphere.

Phosphorothioate oligoribonucleotides are synthesized on an Applied Biosystems Model 380B DNA synthesizer using phosphoroamidite chemistry. A sequence of 24 bases complementary to the nucleotides at position 1 to 24 of SEQ ID NO: 1 is used as the test oligonucleotide. As a control, another (random) sequence is used: 5'-TCA ACT GAC TAG ATG TAC ATG GAC-3'. Following assembly and deprotection, oligonucleotides are ethanol-precipitated twice, dried, and suspended in phosphate buffered saline at the desired concentration. Purity of the oligonucleotides is tested by capillary gel electrophoresis and ion exchange HPLC. The purified oligonucleotides are added to the culture medium at a concentration of 10 µM once per day for seven days.

The addition of the test oligonucleotide for seven days results in significantly reduced expression of human esterase as determined by Western blotting. This effect is not observed with the control oligonucleotide. After 3 to 7 days, the number of cells in the cultures is counted using an automatic cell counter. The number of cells in cultures treated with the test oligonucleotide (expressed as 100%) is compared with the number of cells in cultures treated with the control oligonucleotide. The number of cells in cultures treated with the test oligonucleotide is not more than 30% of control, indicating that the inhibition of human esterase has an anti-proliferative effect on cancer cells.

EXAMPLE 8

In vivo testing of compounds/target validation

Acute Mechanistic Assays

Reduction in Mitogenic Plasma Hormone Levels

This non-tumor assay measures the ability of a compound to reduce either the endogenous level of a circulating hormone or the level of hormone produced in response to a biologic stimulus. Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.). At a predetermined time after administration of test compound, blood plasma is collected. Plasma is assayed for levels of the hormone of interest. If the normal circulating levels of the hormone are too low and/or variable to provide consistent results, the level of the hormone may be elevated by a pre-treatment with a biologic stimulus (i.e., LHRH may be injected i.m. into mice at a dosage of 30 ng/mouse to induce a burst of testosterone synthesis). The timing of plasma collection would be adjusted to coincide with the peak of the induced hormone response. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value $\leq$ 0.05 compared to the vehicle control group.

Hollow Fiber Mechanism of Action Assay

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol, these may include assays for gene expression (bDNA, PCR, or Taqman), or a specific biochemical activity (i.e., cAMP levels. Results are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at $p \leq 0.05$ as compared to the vehicle control group.

Subacute Functional In Vivo *Assays*

Reduction in Mass of Hormone Dependent Tissues

This is another non-tumor assay that measures the ability of a compound to reduce the mass of a hormone dependent tissue (i.e., seminal vesicles in males and uteri in females). Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.) according to a predetermined schedule and for a predetermined duration (i.e., 1 week). At termination of the study, animals are weighed, the target organ is excised, any fluid is expressed, and the weight of the organ is recorded. Blood plasma may also be collected. Plasma may be assayed for levels of a hormone of interest or for levels of test agent. Organ weights may be directly compared or they may be normalized for the body weight of the animal. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value ≤ 0.05 compared to the vehicle control group.

Hollow Fiber Proliferation Assay

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol. Cell proliferation is determined by measuring a marker of cell number (i.e., MTT or LDH). The cell number and change in cell number from the starting inoculum are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at $p \leq 0.05$ as compared to the vehicle control group.

Anti-angiogenesis Models

Corneal Angiogenesis

Hydron pellets with or without growth factors or cells are implanted into a micropocket surgically created in the rodent cornea. Compound administration may be systemic or local (compound mixed with growth factors in the hydron pellet). Corneas are harvested at 7 days post implantation immediately following intracardiac infusion of colloidal carbon and are fixed in 10% formalin. Readout is qualitative scoring and/or image analysis. Qualitative scores are compared by Rank Sum test. Image analysis data is evaluated by measuring the area of neovascularization (in pixels) and group averages are compared by Student's t-test (2 tail). Significance is $p \leq 0.05$ as compared to the growth factor or cells only group.

Matrigel Angiogenesis

Matrigel, containing cells or growth factors, is injected subcutaneously. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Matrigel plugs are harvested at predetermined time point(s) and prepared for readout. Readout is an ELISA-based assay for hemoglobin concentration and/or histological examination (i.e. vessel count, special staining for endothelial surface markers: CD31, factor-8). Readouts are analyzed by Student's t-test, after the variance between groups is compared by an F-test, with significance determined at $p \leq 0.05$ as compared to the vehicle control group.

Primary Antitumor Efficacy

Early Therapy Models

Subcutaneous Tumor

Tumor cells or fragments are implanted subcutaneously on Day 0. Vehicle and/or compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined

schedule starting at a time, usually on Day 1, prior to the ability to measure the tumor burden. Body weights and tumor measurements are recorded 2-3 times weekly. Mean net body and tumor weights are calculated for each data collection day. Anti-tumor efficacy may be initially determined by comparing the size of treated (T) and control (C) tumors on a given day by a Student's t-test, after the variance between groups is compared by an F-test, with significance determined at $p \leq 0.05$. The experiment may also be continued past the end of dosing in which case tumor measurements would continue to be recorded to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is $p \leq 0.05$.

Intraperitoneal/Intracranial Tumor Models

Tumor cells are injected intraperitoneally or intracranially on Day 0. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting on Day 1. Observations of morbidity and/or mortality are recorded twice daily. Body weights are measured and recorded twice weekly. Morbidity/mortality data is expressed in terms of the median time of survival and the number of long-term survivors is indicated separately. Survival times are used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment.

Established Disease Model

Tumor cells or fragments are implanted subcutaneously and grown to the desired size for treatment to begin. Once at the predetermined size range, mice are randomized into treatment groups. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Tumor and body weights are measured and

recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is p value ≤ 0.05 compared to the vehicle control group.

Orthotopic Disease Models

Mammary Fat Pad Assay

Tumor cells or fragments, of mammary adenocarcinoma origin, are implanted directly into a surgically exposed and reflected mammary fat pad in rodents. The fat pad is placed back in its original position and the surgical site is closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Tumor and body weights are measured and recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group.

Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the

median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is p value$\leq$ 0.05 compared to the vehicle control group. In addition, this model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ, or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intraprostatic Assay

Tumor cells or fragments, of prostatic adenocarcinoma origin, are implanted directly into a surgically exposed dorsal lobe of the prostate in rodents. The prostate is externalized through an abdominal incision so that the tumor can be implanted specifically in the dorsal lobe while verifying that the implant does not enter the seminal vesicles. The successfully inoculated prostate is replaced in the abdomen and the incisions through the abdomen and skin are closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the lungs), or measuring the target organ weight (i.e., the regional lymph nodes). The means of these endpoints are compared by Student's t-test after conducting an F-test,

with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intrabronchial Assay

Tumor cells of pulmonary origin may be implanted intrabronchially by making an incision through the skin and exposing the trachea. The trachea is pierced with the beveled end of a 25 gauge needle and the tumor cells are inoculated into the main bronchus using a flat-ended 27 gauge needle with a 90° bend. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the contralateral lung), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intracecal Assay

Tumor cells of gastrointestinal origin may be implanted intracecally by making an abdominal incision through the skin and externalizing the intestine. Tumor cells are inoculated into the cecal wall without penetrating the lumen of the intestine using a 27 or 30 gauge needle. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal

is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the liver), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Secondary (Metastatic) Antitumor Efficacy

Spontaneous Metastasis

Tumor cells are inoculated s.c. and the tumors allowed to grow to a predetermined range for spontaneous metastasis studies to the lung or liver. These primary tumors are then excised. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule which may include the period leading up to the excision of the primary tumor to evaluate therapies directed at inhibiting the early stages of tumor metastasis. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment for both of these endpoints.

Forced Metastasis

Tumor cells are injected into the tail vein, portal vein, or the left ventricle of the heart in experimental (forced) lung, liver, and bone metastasis studies, respectively. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance at $p \leq 0.05$ compared to the vehicle control group in the experiment for both endpoints.

EXAMPLE 9

Diabetes: In vivo testing of compounds/target validation

Glucose Production

Over-production of glucose by the liver, due to an enhanced rate of gluconeogenesis, is the major cause of fasting hyperglycemia in diabetes. Overnight fasted normal rats or mice have elevated rates of gluconeogenesis as do streptozotocin-induced diabetic rats or mice fed ad libitum. Rats are made diabetic with a single intravenous injection of 40 mg/kg of streptozotocin while C57BL/KsJ mice are given 40-60 mg/kg i.p. for 5 consecutive days. Blood glucose is measured from tail-tip blood and then compounds are administered via different routes (p.o., i.p., i.v., s.c.). Blood is collected at various times thereafter and glucose measured. Alternatively, compounds are administered for several days, then the animals are fasted overnight, blood is collected and plasma glucose measured. Compounds that inhibit glucose

production will decrease plasma glucose levels compared to the vehicle-treated control group.

Insulin Sensitivity

Both ob/ob and db/db mice as well as diabetic Zucker rats are hyperglycemic, hyperinsulinemic and insulin resistant. The animals are pre-bled, their glucose levels measured, and then they are grouped so that the mean glucose level is the same for each group. Compounds are administered daily either q.d. or b.i.d. by different routes (p.o., i.p., s.c.) for 7-28 days. Blood is collected at various times and plasma glucose and insulin levels determined. Compounds that improve insulin sensitivity in these models will decrease both plasma glucose and insulin levels when compared to the vehicle-treated control group.

Insulin Secretion

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, compounds are administered by different routes (p.o., i.p., s.c. or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1 g/kg), bled again after 15, 30, 60 and 90 minutes and plasma glucose levels determined. Compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, test compounds which regulate esterase are administered by different routes (p.o., i.p., s.c., or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Test compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1 g/kg), bled again after 15, 30, 60, and 90 minutes and plasma glucose levels determined. Test compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

EXAMPLE 10

In vivo testing of compounds/target validation

Pain

Acute pain. Acute pain is measured on a hot plate mainly in rats. Two variants of hot plate testing are used: In the classical variant animals are put on a hot surface (52 to 56°C) and the latency time is measured until the animals show nocifensive behavior, such as stepping or foot licking. The other variant is an increasing temperature hot plate where the experimental animals are put on a surface of neutral temperature. Subsequently this surface is slowly but constantly heated until the animals begin to lick a hind paw. The temperature which is reached when hind paw licking begins is a measure for pain threshold.

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Persistent pain. Persistent pain is measured with the formalin or capsaicin test, mainly in rats. A solution of 1 to 5% formalin or 10 to 100 µg capsaicin is injected into one hind paw of the experimental animal. After formalin or capsaicin application the animals show nocifensive reactions like flinching, licking and biting of the affected paw. The number of nocifensive reactions within a time frame of up to 90 minutes is a measure for intensity of pain.

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to formalin or capsaicin administration.

Neuropathic pain. Neuropathic pain is induced by different variants of unilateral sciatic nerve injury mainly in rats. The operation is performed under anesthesia. The first variant of sciatic nerve injury is produced by placing loosely constrictive ligatures around the common sciatic nerve. The second variant is the tight ligation of about the half of the diameter of the common sciatic nerve. In the next variant, a group of models is used in which tight ligations or transections are made of either the L5 and L6 spinal nerves, or the L% spinal nerve only. The fourth variant involves an axotomy of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact whereas the last variant comprises the axotomy of only the tibial branch leaving the sural and common nerves uninjured. Control animals are treated with a sham operation.

Postoperatively, the nerve injured animals develop a chronic mechanical allodynia, cold allodynioa, as well as a thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA; Electronic von Frey

System, Somedic Sales AB, Hörby, Sweden). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy), or by means of a cold plate of 5 to 10°C where the nocifensive reactions of the affected hind paw are counted as a measure of pain intensity. A further test for cold induced pain is the counting of nocifensive reactions, or duration of nocifensive responses after plantar administration of acetone to the affected hind limb. Chronic pain in general is assessed by registering the circadanian rhythms in activity (Surjo and Arndt, Universität zu Köln, Cologne, Germany), and by scoring differences in gait (foot print patterns; FOOTPRINTS program, Klapdor et al., 1997. A low cost method to analyze footprint patterns. J. Neurosci. Methods 75, 49-54).

Compounds are tested against sham operated and vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Inflammatory Pain. Inflammatory pain is induced mainly in rats by injection of 0.75 mg carrageenan or complete Freund's adjuvant into one hind paw. The animals develop an edema with mechanical allodynia as well as thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, ITC Inc.-Life Science Instruments, Woodland Hills, SA, USA). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy, Paw thermal stimulator, G. Ozaki, University of California, USA). For edema measurement two methods are being used. In the first method, the animals are sacrificed and the affected hindpaws sectioned and weighed. The second method comprises differences in paw volume by measuring water displacement in a plethysmometer (Ugo Basile, Comerio, Italy).

Compounds are tested against uninflamed as well as vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Diabetic neuropathic pain. Rats treated with a single intraperitoneal injection of 50 to 80 mg/kg streptozotocin develop a profound hyperglycemia and mechanical allodynia within 1 to 3 weeks. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA).

Compounds are tested against diabetic and non-diabetic vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Parkinson's disease

6-Hydroxydopamine (6-OH-DA) Lesion. Degeneration of the dopaminergic nigrostriatal and striatopallidal pathways is the central pathological event in Parkinson's disease. This disorder has been mimicked experimentally in rats using single/sequential unilateral stereotaxic injections of 6-OH-DA into the medium forebrain bundle (MFB).

Male Wistar rats (Harlan Winkelmann, Germany), weighing 200±250 g at the beginning of the experiment, are used. The rats are maintained in a temperature- and humidity-controlled environment under a 12 h light/dark cycle with free access to food and water when not in experimental sessions. The following in vivo protocols are approved by the governmental authorities. All efforts are made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

Animals are administered pargyline on the day of surgery (Sigma, St. Louis, MO, USA; 50 mg/kg i.p.) in order to inhibit metabolism of 6-OHDA by monoamine oxidase and desmethylimipramine HCl (Sigma; 25 mg/kg i.p.) in order to prevent uptake of 6-OHDA by noradrenergic terminals. Thirty minutes later the rats are anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic frame.

In order to lesion the DA nigrostriatal pathway 4 µl of 0.01% ascorbic acid-saline containing 8 µg of 6-OHDA HBr (Sigma) are injected into the left medial fore-brain bundle at a rate of 1 µl/min (2.4 mm anterior, 1.49 mm lateral, -2.7 mm ventral to Bregma and the skull surface). The needle is left in place an additional 5 min to allow diffusion to occur.

Stepping Test: Forelimb akinesia is assessed three weeks following lesion placement using a modified stepping test protocol. In brief, the animals are held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. One paw is touching the table, and is then moved slowly sideways (5 s for 1 m), first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand direction of movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated three times on three consecutive days, after an initial training period of three days prior to the first testing. Forehand adjusted stepping reveals no consistent differences between lesioned and healthy control animals. Analysis is therefore restricted to backhand adjusted stepping.

Balance Test. Balance adjustments following postural challenge are also measured during the stepping test sessions. The rats are held in the same position as described in the stepping test and, instead of being moved sideways, tilted by the experimenter towards the side of the paw touching the table. This maneuver results in loss of balance and the ability of the rats to regain balance by forelimb movements is scored on a scale ranging from 0 to 3. Score 0 is given for a normal forelimb placement. When the forelimb movement is delayed but recovery of postural balance detected, score 1 is given. Score 2 represents a clear, yet insufficient, forelimb reaction, as evidenced by muscle contraction, but lack of success in recovering balance, and score 3 is given for no reaction of movement. The test is repeated three times a day on each side for three consecutive days after an initial training period of three days prior to the first testing.

Staircase Test (Paw Reaching). A modified version of the staircase test is used for evaluation of paw reaching behavior three weeks following primary and secondary lesion placement. Plexiglass test boxes with a central platform and a removable staircase on each side are used. The apparatus is designed such that only the paw on the same side at each staircase can be used, thus providing a measure of independent forelimb use. For each test the animals are left in the test boxes for 15 min. The double staircase is filled with 7 x 3 chow pellets (Precision food pellets, formula: P, purified rodent diet, size 45 mg; Sandown Scientific) on each side. After each test the number of pellets eaten (successfully retrieved pellets) and the number of pellets taken (touched but dropped) for each paw and the success rate (pellets eaten/pellets taken) are counted separately. After three days of food deprivation (12 g per animal per day) the animals are tested for 11 days. Full analysis is conducted only for the last five days.

MPTP treatment. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes degeneration of mesencephalic dopaminergic (DAergic) neurons in rodents, non-human primates, and humans and, in so doing, reproduces many of the symptoms of Parkinson's disease. MPTP leads to a marked decrease in the levels of dopamine and its metabolites, and in the number of dopaminergic terminals in the striatum as well as severe loss of the tyrosine hydroxylase (TH)-immunoreactive cell bodies in the substantia nigra, pars compacta.

In order to obtain severe and long-lasting lesions, and to reduce mortality, animals receive single injections of MPTP, and are then tested for severity of lesion 7–10 days later. Successive MPTP injections are administered on days 1, 2 and 3. Animals receive application of 4 mg/kg MPTP hydrochloride (Sigma) in saline once daily. All injections are intraperitoneal (i.p.) and the MPTP stock solution is frozen between injections. Animals are decapitated on day 11.

Immunohistology. At the completion of behavioral experiments, all animals are anaesthetized with 3 ml thiopental (1 g/40 ml i.p., Tyrol Pharma). The mice are perfused transcardially with 0.01 M PBS (pH 7.4) for 2 min, followed by 4% paraformaldehyde (Merck) in PBS for 15 min. The brains are removed and placed in 4% paraformaldehyde for 24 h at 4°C. For dehydration they are then transferred to a 20% sucrose (Merck) solution in 0.1 M PBS at 4°C until they sink. The brains are frozen in methylbutan at -20°C for 2 min and stored at -70°C. Using a sledge microtome (mod. 3800-Frigocut, Leica), 25 µm sections are taken from the genu of the corpus callosum (AP 1.7 mm) to the hippocampus (AP 21.8 mm) and from AP 24.16 to AP 26.72. Forty-six sections are cut and stored in assorters in 0.25 M Tris buffer (pH 7.4) for immunohistochemistry.

A series of sections is processed for free-floating tyrosine hydroxylase (TH) immunohistochemistry. Following three rinses in 0.1 M PBS, endogenous peroxidase activity is quenched for 10 min in 0.3% H_2O_2 ±PBS. After rinsing in PBS, sections are preincubated in 10% normal bovine serum (Sigma) for 5 min as blocking agent and transferred to either primary anti-rat TH rabbit antiserum (dilution 1:2000).

Following overnight incubation at room temperature, sections for TH immunoreactivity are rinsed in PBS (2 x10 min) and incubated in biotinylated anti-rabbit immunoglobulin G raised in goat (dilution 1:200) (Vector) for 90 min, rinsed repeatedly and transferred to Vectastain ABC (Vector) solution for 1 h. 3,.3' -Diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.1 M PBS, supplemented with 0.005% H_2O_2, serves as chromogen in the subsequent visualization reaction. Sections are mounted on to gelatin-coated slides, left to dry overnight, counterstained with hematoxylin dehydrated in ascending alcohol concentrations and cleared in butylacetate. Coverslips are mounted on entellan.

Rotarod Test. We use a modification of the procedure described by Rozas and Labandeira-Garcia (1997), with a CR-1 Rotamex system (Columbus Instruments,

Columbus, OH) comprising an IBM-compatible personal computer, a CIO-24 data acquisition card, a control unit, and a four-lane rotarod unit. The rotarod unit consists of a rotating spindle (diameter 7.3 cm) and individual compartments for each mouse. The system software allows preprogramming of session protocols with varying rotational speeds (0–80 rpm). Infrared beams are used to detect when a mouse has fallen onto the base grid beneath the rotarod. The system logs the fall as the end of the experiment for that mouse, and the total time on the rotarod, as well as the time of the fall and all the set-up parameters, are recorded. The system also allows a weak current to be passed through the base grid, to aid training.

Dementia

The object recognition task. The object recognition task has been designed to assess the effects of experimental manipulations on the cognitive performance of rodents. A rat is placed in an open field, in which two identical objects are present. The rats inspects both objects during the first trial of the object recognition task. In a second trial, after a retention interval of for example 24 hours, one of the two objects used in the first trial, the 'familiar' object, and a novel object are placed in the open field. The inspection time at each of the objects is registered. The basic measures in the OR task is the time spent by a rat exploring the two object the second trial. Good retention is reflected by higher exploration times towards the novel than the 'familiar' object.

Administration of the putative cognition enhancer prior to the first trial predominantly allows assessment of the effects on acquisition, and eventually on consolidation processes. Administration of the testing compound after the first trial allows to assess the effects on consolidation processes, whereas administration before the second trial allows to measure effects on retrieval processes.

The passive avoidance task. The passive avoidance task assesses memory performance in rats and mice. The inhibitory avoidance apparatus consists of a

two-compartment box with a light compartment and a dark compartment. The two compartments are separated by a guillotine door that can be operated by the experimenter. A threshold of 2 cm separates the two compartments when the guillotine door is raised. When the door is open, the illumination in the dark compartment is about 2 lux. The light intensity is about 500 lux at the center of the floor of the light compartment.

Two habituation sessions, one shock session, and a retention session are given, separated by inter-session intervals of 24 hours. In the habituation sessions and the retention session the rat is allowed to explore the apparatus for 300 sec. The rat is placed in the light compartment, facing the wall opposite to the guillotine door. After an accommodation period of 15 sec. the guillotine door is opened so that all parts of the apparatus can be visited freely. Rats normally avoid brightly lit areas and will enter the dark compartment within a few seconds.

In the shock session the guillotine door between the compartments is lowered as soon as the rat has entered the dark compartment with its four paws, and a scrambled 1 mA footshock is administered for 2 sec. The rat is removed from the apparatus and put back into its home cage. The procedure during the retention session is identical to that of the habituation sessions.

The step-through latency, that is the first latency of entering the dark compartment (in sec.) during the retention session is an index of the memory performance of the animal; the longer the latency to enter the dark compartment, the better the retention is. A testing compound in given half an hour before the shock session, together with 1 $mg*kg^{-1}$ scopolamine. Scopolamine impairs the memory performance during the retention session 24 hours later. If the test compound increases the enter latency compared with the scopolamine-treated controls, is likely to possess cognition enhancing potential.

The Morris water escape task. The Morris water escape task measures spatial orientation learning in rodents. It is a test system that has extensively been used to investigate the effects of putative therapeutic on the cognitive functions of rats and mice. The performance of an animal is assessed in a circular water tank with an escape platform that is submerged about 1 cm below the surface of the water. The escape platform is not visible for an animal swimming in the water tank. Abundant extra-maze cues are provided by the furniture in the room, including desks, computer equipment, a second water tank, the presence of the experimenter, and by a radio on a shelf that is playing softly.

The animals receive four trials during five daily acquisition sessions. A trial is started by placing an animal into the pool, facing the wall of the tank. Each of four starting positions in the quadrants north, east, south, and west is used once in a series of four trials; their order is randomized. The escape platform is always in the same position. A trial is terminated as soon as the animal had climbs onto the escape platform or when 90 seconds have elapsed, whichever event occurs first. The animal is allowed to stay on the platform for 30 seconds. Then it is taken from the platform and the next trial is started. If an animal did not find the platform within 90 seconds it is put on the platform by the experimenter and is allowed to stay there for 30 seconds. After the fourth trial of the fifth daily session, an additional trial is given as a probe trial: the platform is removed, and the time the animal spends in the four quadrants is measured for 30 or 60 seconds. In the probe trial, all animals start from the same start position, opposite to the quadrant where the escape platform had been positioned during acquisition.

Four different measures are taken to evaluate the performance of an animal during acquisition training: escape latency, traveled distance, distance to platform, and swimming speed. The following measures are evaluated for the probe trial: time (s) in quadrants and traveled distance (cm) in the four quadrants. The probe trial provides additional information about how well an animal learned the position of the escape platform. If an animal spends more time and swims a longer distance in the

quadrant where the platform had been positioned during the acquisition sessions than in any other quadrant, one concludes that the platform position has been learned well.

In order to assess the effects of putative cognition enhancing compounds, rats or mice with specific brain lesions which impair cognitive functions, or animals treated with compounds such as scopolamine or MK-801, which interfere with normal learning, or aged animals which suffer from cognitive deficits, are used.

The T-maze spontaneous alternation task. The T-maze spontaneous alternation task (TeMCAT) assesses the spatial memory performance in mice. The start arm and the two goal arms of the T-maze are provided with guillotine doors which can be operated manually by the experimenter. A mouse is put into the start arm at the beginning of training. The guillotine door is closed. In the first trial, the 'forced trial', either the left or right goal arm is blocked by lowering the guillotine door. After the mouse has been released from the start arm, it will negotiate the maze, eventually enter the open goal arm, and return to the start position, where it will be confined for 5 seconds, by lowering the guillotine door. Then, the animal can choose freely between the left and right goal arm (all guillotine-doors opened) during 14 'free choice' trials. As soon a the mouse has entered one goal arm, the other one is closed. The mouse eventually returns to the start arm and is free to visit whichever go alarm it wants after having been confined to the start arm for 5 seconds. After completion of 14 free choice trials in one session, the animal is removed from the maze. During training, the animal is never handled.

The percent alternations out of 14 trials is calculated. This percentage and the total time needed to complete the first forced trial and the subsequent 14 free choice trials (in s) is analyzed. Cognitive deficits are usually induced by an injection of scopolamine, 30 min before the start of the training session. Scopolamine reduced the per-cent alternations to chance level, or below. A cognition enhancer, which is always administered before the training session, will at least partially, antagonize the scopolamine-induced reduction in the spontaneous alternation rate.

EXAMPLE 11

Identification of test compound efficacy in an animal model of COPD

A/J mice are exposed to the smoke from 2 unfiltered cigarettes per day for 6 days per week for 14 weeks. Non-smoking, age-matched animals are used as controls. Animals are orally dosed with test compound or vehicle 1 hour before and 7 hours after smoke exposure. This twice-daily dosing regime is continued throughout the smoke exposure period. On day 7 of the weekly exposure, animals are given only 1 dose of test compound and are not exposed to cigarette smoke.

After the smoke exposure period, the mice are killed, their lungs inflated with phosphate-buffered formalin via their trachea, and then the lungs and heart are removed *en bloc* and fixed at 4°C for 48 hours. The lungs are then prepared for paraffin wax sectioning, and 4 mm sections are cut and mounted on glass slides. Sections are then stained with haematoxylin and eosin. Morphometric analysis of lung sections is done by calculation of the Linear Mean Intercept (LMI) parameter using a semi-automated computer image analysis system. Each slide (1 per mouse) contains several sections originating from multiple lobes. Twelve non-overlapping areas (each area covering 1.53 x 10-3 cm^2) are randomly selected for LMI analysis. The 12 areas cover a minimum of two lobes per slide. Non-parenchymal components (airways, blood vessels) are excluded from the analysis to prevent artifactual error. The mean intercept length is calculated for each mouse. Development of emphysema is seen as an increase in LMI.

LMI data are expressed as the median and statistical comparisons are done using the non-parametric Mann-Witney U-test. A 'p' value of <=0.05 is considered to be statistically significant. The potency of a test compound is evaluated by comparison of the tobacco smoke induced increase in LMI in animals dosed with either the test compound or just the vehicle used for administration of the compound.

EXAMPLE 12

Identification of test compound efficacy in an in vitro functional test relevant to COPD

The potency of test compounds is evaluated by measuring the inhibition of elastolysis induced by human alveolar macrophages. The cells are isolated from bronchoalveolar lavage samples taken from non-smokers, disease-free smokers, and smokers with COPD. Macrophage suspensions are added to test wells coated with tritiated elastin and incubated at 37°C for 3h to allow adherence of the cells. The wells are then carefully washed to remove non-adherent cells and fresh medium is added to each well. The cells are incubated at 37°C for up to 72 hours in the presence or absence of test compound. Every 24 hours the medium in each well is removed for analysis and replaced by fresh medium. Radioactivity released into the medium is measured by liquid scintillation counting and the rate of elastin degradation is calculated. The potency of a test compound is evaluated by comparing the rate of elastolysis measured with cells incubated in the presence or absence of the compound.

EXAMPLE 13

In vivo testing of compounds/target validation

Mouse anti-CD3 induced cytokine production model

BALB/c mice are injected with a single intravenous injection of 10 µg of 145-2C11 (purified hamster anti-mouse CD3ε monoclonal antibodies, PHARMINGEN). A test compound is administered intraperitoneally 60 min prior to the anti-CD3 mAb injection. Blood is collected 90 minutes after the antibody injection. Serum is

obtained by centrifugation at 3000 r.p.m. for 10 min. IL-2 and IL-4 levels in the serum are determined by an ELISA.

Mouse anti-IgD induced IgE production model

BALB/c mice are injected intravenously with 0.8 mg of purified goat anti-mouse IgD antibody or PBS (defined as day 0). Compound is administered intraperitoneally from day 0 to day 6. On day 7 blood is collected and serum is obtained by centrifugation at 3000 r.p.m. for 10 min. Serum total levels of IgE are determined by YAMASA's ELISA kit and their Ig subtypes are done by an Ig ELISA KIT (Rougier Biotech's, Montreal, Canada).

Mouse LPS-induced TNF-α production model

BALB/c mice are injected intraperitoneally with LPS (200 μg/mouse). Compound is administered intraperitoneally 1 hr before the LPS injection. Blood is collected at 90 min post-LPS injection and plasma is obtained. TNF-α concentration in the sample is determined using an ELISA kit.

Mouse eotaxin-induced eosinophilia model

BALB/c mice are injected intradermally with a 2.5 ml of air on days –6 and –3 to prepare airpouch. On day 0 compound is administered intraperitoneally 60 min before eotaxin injection (3 μg/mouse, i.d.). IL-5 (300 ng/mouse) is injected intravenously 30 min before the eotaxin injection. After 4 hr of the eotaxin injection leukocytes in exudate is collected and the number of total cells is counted. The differential cell counts in the exudate are performed by staining with May-Grunwald Gimsa solution.

Mouse D10 cell transfer model

D10.G4.1 cells (1 x 10^7 cells/mouse) containing 2 mg of conalbumin in saline is administered i.v. to AKR mice. After 6 hr blood is collected and serum is obtained by centrifugation at 3000 r.p.m. for 10min. IL-4 and IL-5 level in serum are determined by ELISA kits. Compound is administered intraperitoneally at –4 and +1 hr after these cells injection.

Passive cutaneous anaphylaxis (PCA) test in rats

6 Weeks old male Wistar rats are sensitized intradermally (i.d.) on their shaved backs with 50 µl of 0.1 µg/ml mouse anti-DNP IgE monoclonal antibody (SPE-7) under a light anesthesia. After 24 hours, the rats are challenged intravenously with 1 ml of saline containing 0.6 mg DNP-BSA (30) (LSL CO., LTD) and 0.005 g of Evans blue. Compounds are injected intraperitoneally (i.p.) 0.5 hr prior to antigen injection. Rats without the sensitization, challenge, and compound treatment are used for a blank (control) and rats with sensitization, challenge and vehicle treatment are used to determine a value without inhibition. Thirty min after the challenge, the rats are killed, and the skin of the back is removed. Evans blue dye in the skin is extracted in formamide overnight at 63°C. Then an absorbance at 620 nm is measured to obtain the optical density of the leaked dye.

Percent inhibition of PCA with a compound is calculated as follows:

% inhibition = {(mean vehicle value – sample value)/(mean vehicle value – mean control value)} x 100

Anaphylactic bronchoconstriction in rats

6 Weeks old male Wistar rats are sensitized intravenously (i.v.) with 10 µg mouse anti-DNP IgE, SPE-7, and 1 days later, the rats are challenged intravenously with

0.3 ml of saline containing 1.5 mg DNP-BSA (30) under anesthesia with urethane (1000 mg/kg, i.p.) and gallamine (50 mg/kg, i.v.). The trachea is cannulated for artificial respiration (2 ml / stroke, 70 strokes / min). Pulmonary inflation pressure (PIP) is recorded through a side-arm of cannula connected to pressure transducer. Change in PIP reflects change of both resistance and compliance of the lungs. To evaluate the drugs, each drug is given i.v. 5 min before challenge.

EXAMPLE 14

Bladder outlet obstruction model

Wistar rats (200~250 g / Charles River Japan) are anesthetized intraperitoneally with ketamine. The abdomen is opened through a midline incision and the bladder and the proximal urethra are exposed. A constant degree of urethral obstruction is produced by tying a ligature around the urethra and a catheter with an outer diameter of 1 mm. The abdominal well is closed and the animals allowed to recover.

After 6 weeks, the rats are anesthetized with ketamine, and the ligature around the urethra is carefully removed to normalize the outlet resistance and enable repetitive micturition. A polyethylene catheter is implanted in the bladder through the dome, and exteriorized at the scapular level. Animals are then allowed to recover for at least 48 hours.

Cytometric investigation is performed without anesthesia two days after bladder catheter implantation in control and obstructed animals. The bladder catheter was connected via a T-tube to a strain gauge and a microinjection pump. The conscious rats are held under partial restraint in a restraining device. Warmed saline is infused into the bladder at a rate of 3 ml/hr for control and obstructed animals. The rate of infusion is increased from 3 to 10 ml/hr to obtain similar interval times between micturitions in obstructed and control rats. Overactivity of the obstructed bladders is assessed by measuring the cystometric parameters such as basal pressure, peak

micturition pressure, threshold pressure, micturition interval, amplitude and frequency of spontaneous activity and micturition slope. Lluel *et al.*, *J. Urol. 160*, 2253-57, 1998.

A test compound is dissolved in an appropriate vehicle, such as a mixture of ethanol, Tween 80 (ICN Biomedicals Inc.), and saline (1:1:8, v/v/v), is administered intravenously through the catheter.

Organ bath assay for measuring agonist-induced contraction of prostate

An organ bath assay is employed to measure the agonist-induced contraction of prostate for assessing the biological activity of test compounds (*i.e.*, drug candidates). Male Wistar rats (200~250 g / Charles River Japan) are anesthetized with ether and sacrificed by dislocating the necks. The whole prostate is excised and placed in oxygenated Modified Krebs-Henseleit solution (pH 7.4) of the following composition (112mM NaCl, 5.9mM KCl, 1.2mM $MgCl_2$, 1.2mM NaH_2PO_4, 2mM $CaCl_2$, 2.5mM $NaHCO_3$, 12mM glucose). Ventricle prostate lobes were dissected into several strips depending on the size of prostate. Prostate strips are equilibrated for 60 min in organ bath chambers before any stimulation.

Isometric tension is recorded under an appropriate load. Contractile response to adrenergic agonists or electric field stimulation is determined several times until reproducible responses are obtained. Test compounds are pre-incubated prior to the agonistic or electric stimulation. The ratio of each contraction to the negative control is calculated and the effect of the test compounds on the prostate contraction is evaluated.

Organ bath assay for measuring agonist-induced contraction of urinary bladder

An organ bath assay is employed to measure the agonist-induced contraction of urinary bladder for assessing the biological activity of test compounds (*i.e.*, drug

candidates). Male Wistar rats (200~250 g / Charles River Japan) are anesthetized with ether and sacrificed by dislocating the necks. The whole urinary bladder is excised and placed in oxygenated Modified Krebs-Henseleit solution (pH 7.4) of the following composition (112 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 12 mM glucose).

Isometric tension is recorded under an appropriate load using longitudinal strips of rat detrusor muscle. Bladder strips are equilibrated for 60 minutes before each stimulation. Contractile response to 80 mM KCl is determined at 15 minute intervals until reproducible responses are obtained. The response to KCl is used as an internal standard to evaluate the effect of test compounds.

The effects of test compounds are investigated by incubating the strips with compounds for 30 minutes prior to stimulation with an appropriate agonist or electrical stimulation. One of the preparations made from the same animal serves as a control, while others are used for evaluating test compounds. The ratio of each contraction to the internal standard (*e.g.*, a KCl-induced contraction) is calculated, and the effects of the test compounds on the contraction are evaluated.

EXAMPLE 15

In vivo testing of compounds/target validation

(1) Animals. Female Sprague-Dawley rats (200~250 g / Charles River Japan) are used.

(2) Catheter implantation. Rats are anesthetized by intraperitoneal administration of urethane (Sigma) at 1.25 g/kg. The abdomen is opened through a midline incision, and a polyethylene catheter (BECTON DICKINSON, PE50) is implanted into the bladder through the dome. In parallel, the inguinal region

is incised, and a polyethylene catheter (BECTON DICKINSON, PE50) filled with saline (Otsuka) is inserted into a femoral vein.

(3) Investigation of bladder contraction. The bladder is filled via the catheter by incremental volume of saline until spontaneous bladder contractions occur. The intravesicular pressure is measured a pressure transducer and displayed continuously on a chart recorder. The activity of test compounds is assessed after intravenous administration through a polyethylene cannula inserted into the femoral vein.

Measurement of bladder cystometry in conscious rats

(1) Animals. Female Sprague-Dawley rats (200–250 g / Charles River Japan) are used.

(2) Catheter implantation. Rats are anesthetized by intramuscular administration of ketamine (75 mg/kg) and xylazine (15 mg/kg). The abdomen is opened through a midline incision, and a polyethylene catheter (BECTON DICKINSON, PE50) is implanted into the bladder through the dome. The catheter is tunneled through subcutis of the animal by needle (14G) to neck. In parallel, the inguinal region is incised, and a polyethylene catheter (BECTON DICKINSON, PE50) filled with saline (Otsuka) is inserted into a femoral vein. The catheter is tunneled through subcutis of the animal by needle to neck.

(3) Cystometric investigation. The bladder catheter is connected via T-tube to a pressure transducer (Viggo-Spectramed Pte Ltd, DT-XXAD) and a micro-injection pump (TERUMO). Saline is infused at room temperature into the bladder at a rate of 10 ml/hr. Intravesicular pressure is recorded continuously on a chart pen recorder (Yokogawa). At least three reproducible micturition cycles are recorded before a test compound administration.

(4) Administration of test compounds. A test compound dissolved in the mixture of ethanol, Tween 80 (ICN Biomedicals Inc.) and saline (1 : 1 : 8, v/v/v) is administered intravenously through the catheter.

EXAMPLE 16

In vivo testing of compounds/target validation

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killed, and the skin of the back is removed. Evans blue dye in the skin is extracted in formamide overnight at 63°C. Then an absorbance at 620 nm is measured to obtain the optical density of the leaked dye.

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EXAMPLE 17

Expression profiling

Total cellular RNA was isolated from cells by one of two standard methods:
1) guanidine isothiocyanate/cesium chloride density gradient centrifugation [Kellogg *et al.* (1990)]; or with the Tri-Reagent protocol according to the manufacturer's specifications (Molecular Research Center, Inc., Cincinatti, Ohio). Total RNA prepared by the Tri-reagent protocol was treated with DNAse I to remove genomic DNA contamination.

For relative quantitation of the mRNA distribution, total RNA from each cell or tissue source was first reverse transcribed. Eighty-five µg of total RNA was reverse transcribed using 1 µmole random hexamer primers, 0.5 mM each of dATP, dCTP, dGTP and dTTP (Qiagen, Hilden, Germany) and 3000 U RnaseQut (Invitrogen, Groningen, Netherlands) in a final volume of 680 µl. The first strand synthesis buffer and Omniscript reverse transcriptase (2 u/µl) were obtained from (Qiagen, Hilden, Germany). The reaction was incubated at 37°C for 90 minutes and cooled on ice. The volume was adjusted to 6800 µl with water, yielding a final concentration of 12.5 ng/µl of starting RNA.

For relative quantitation of the distribution of mRNA in cells and tissues the Perkin Elmer ABI Prism R™ 7700 Sequence Detection system or Biorad iCycler was used according to the manufacturer's specifications and protocols. PCR reactions were set up to quantitate expression of the test gene and the housekeeping genes HPRT (hypoxanthine phosphoribosyltransferase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ß -actin, and others. Forward and reverse primers and probes were designed using the Perkin Elmer ABI Primer Express™ software and were synthesized by TibMolBiol (Berlin, Germany). The forward primer sequence was: Primer1 gccgtcgaggtcttcttctc. The reverse primer sequence was Primer2 cgagaagagcagcgtgtagc. Probe1 aaccggctcggctgcatgttc, labeled with FAM (carboxy-fluorescein succinimidyl ester) as the reporter dye and TAMRA (carboxytetra-methylrhodamine) as the quencher, was used as a probe. The following reagents were prepared in a total of 25 µl : 1x TaqMan buffer A, 5.5 mM $MgCl_2$, 200 nM of dATP, dCTP, dGTP, and dUTP, 0.025 U/µl AmpliTaq Gold™, 0.01 U/µl AmpErase, and Probe1 aaccggctcggctgcatgttc, forward and reverse primers each at 200 nM, 200 nM , FAM/TAMRA-labeled probe, and 5 µ l of template cDNA. Thermal cycling parameters were 2 min at 50°C, followed by 10 min at 95°C, followed by 40 cycles of melting at 95°C for 15 sec and annealing/extending at 60°C for 1 min.

Calculation of corrected CT values

The CT (threshold cycle) value is calculated as described in the "Quantitative determination of nucleic acids" section. The CF-value (factor for threshold cycle correction) is calculated as follows:

1. PCR reactions were set up to quantitate the housekeeping genes (HKG) for each cDNA sample.

2. CT_{HKG}-values (threshold cycle for housekeeping gene) were calculated as described in the "Quantitative determination of nucleic acids" section.

3. CT_{HKG}-mean values (CT mean value of all HKG tested on one cDNAs) of all HKG for each cDNA are calculated (n = number of HKG):

   CT_{HKG-n}-mean value = (CT_{HKG1}-value + CT_{HKG2}-value + ... + CT_{HKG-n}-value) / n

4. CT_{pannel} mean value (CT mean value of all HKG in all tested cDNAs) = (CT_{HKG1}-mean value + CT_{HKG2}-mean value + ...+ CT_{HKG-y}-mean value) / y (y = number of cDNAs)

5. CF_{cDNA-n} (correction factor for cDNA n) = CT_{pannel}-mean value - CT_{HKG-n}-mean value

6. CT_{cDNA-n} (CT value of the tested gene for the cDNA n) + CF_{cDNA-n} (correction factor for cDNA n) = $CT_{cor-cDNA-n}$ (corrected CT value for a gene on cDNA n)

Calculation of relative expression

Definition: highest $CT_{cor-cDNA-n} \neq 40$ is defined as $CT_{cor-cDNA}$ [high]

Relative Expression = $2^{(CTcor-cDNA[high] - CTcor-cDNA-n)}$

The following tissues were tested: fetal heart, heart, pericardium, heart atrium (right), heart atrium (left), heart ventricle (left), heart ventricle (right), heart apex, Purkinje fibers, interventricular septum, fetal aorta, aorta, artery, coronary artery, pulmonary artery, carotid artery, mesenteric artery, vein, pulmonic valve, coronary artery smooth muscle primary cells, HUVEC cells, skin, adrenal gland, thyroid, thyroid tumor, pancreas, pancreas liver cirrhosis, esophagus, esophagus tumor, stomach, stomach tumor, colon, colon tumor, small intestine, ileum, ileum tumor, ileum chronic inflammation, rectum, salivary gland, fetal liver, liver, liver cirrhosis, liver tumor, HEP G2 cells, leukocytes (peripheral blood), Jurkat (T-cells), bone marrow, erythrocytes, lymph node, thymus, thrombocytes, bone marrow stromal cells, bone marrow CD71+ cells, bone marrow CD33+ cells, bone marrow CD34+ cells, bone marrow CD15+ cells, cord blood CD71+ cells, cord blood CD34+ cells, neutrophils cord blood, neutrophils peripheral blood, spleen, spleen liver cirrhosis, skeletal muscle, adipose, fetal brain, brain, Alzheimer brain, cerebellum, cerebellum (right), cerebellum (left), cerebral cortex, Alzheimer cerebral cortex, frontal lobe, Alzheimer brain frontal lobe, occipital lobe, parietal lobe, temporal lobe, precentral gyrus, postcentral gyrus, tonsilla cerebelli, vermis cerebelli, pons, substantia nigra, cerebral meninges, cerebral peduncles, corpus callosum, hippocampus, thalamus, dorsal root ganglia, spinal cord, neuroblastoma SK-N-MC cells, neuroblastoma SH-SY5Y cells, neuroblastoma IMR32 cells, glial tumor H4 cells, glial tumor H4 cells + APP, HEK CNS, HEK CNS + APP, retina, fetal lung, fetal lung fibroblast IMR-90 cells, fetal lung fibroblast MRC-5 cells, lung, lung right upper lobe, lung right mid lobe, lung right lower lobe, lung lupus disease, lung tumor, lung COPD, trachea, cervix, testis, HeLa cells (cervix tumor), placenta, uterus, uterus tumor, ovary, ovary tumor, breast, breast tumor, MDA MB 231 cells (breast tumor), mammary gland, prostate, prostate BPH, bladder, ureter, penis, corpus cavernosum, fetal kidney, kidney, kidney tumor, and HEK 293 cells.

The results are shown in Table 1.

Tissue	Relative Expression
fetal heart	151
heart	90
pericardium	103
heart atrium (right)	84
heart atrium (left)	129
heart ventricle (left)	11
heart ventricle (right)	9
heart apex	118
Purkinje fibers	127
interventricular septum	67
fetal aorta	1
aorta	2
artery	4
coronary artery	75
pulmonary artery	1
carotid artery	1
mesenteric artery	5
vein	3
pulmonic valve	154
coronary artery smooth muscle primary cells	120
HUVEC cells	82
skin	407
adrenal gland	102
thyroid	142
thyroid tumor	164
pancreas	92
pancreas liver cirrhosis	141

Tissue	Relative Expression
esophagus	30
esophagus tumor	1323
stomach	129
stomach tumor	734
colon	1520
colon tumor	803
small intestine	294
ileum	690
ileum tumor	372
ileum chronic inflammation	62
rectum	592
salivary gland	212
fetal liver	18
liver	66
liver cirrhosis	498
liver tumor	343
HEP G2 cells	184
leukocytes (peripheral blood)	71
Jurkat (T-cells)	61
bone marrow	25
erythrocytes	14
lymph node	29
thymus	56
thrombocytes	25
bone marrow stromal cells	25
bone marrow CD71+ cells	9
bone marrow CD33+ cells	17

Tissue	Relative Expression
bone marrow CD34+ cells	48
bone marrow CD15+ cells	5
cord blood CD71+ cells	9
cord blood CD34+ cells	146
neutrophils cord blood	77
neutrophils peripheral blood	118
spleen	138
spleen liver cirrhosis	207
skeletal muscle	24
adipose	61
fetal brain	755
brain	313
Alzheimer brain	474
cerebellum	44
cerebellum (right)	324
cerebellum (left)	362
cerebral cortex	335
Alzheimer cerebral cortex	271
frontal lobe	360
Alzheimer brain frontal lobe	352
occipital lobe	280
parietal lobe	320
temporal lobe	345
precentral gyrus	331
postcentral gyrus	14
tonsilla cerebelli	261
vermis cerebelli	455

Tissue	Relative Expression
pons	205
substantia nigra	798
cerebral meninges	111
cerebral peduncles	252
corpus callosum	271
hippocampus	247
thalamus	100
dorsal root ganglia	98
spinal cord	298
neuroblastoma SK-N-MC cells	51
neuroblastoma SH-SY5Y cells	534
neuroblastoma IMR32 cells	271
glial tumor H4 cells	226
glial tumor H4 cells + APP	135
HEK CNS	765
HEK CNS + APP	1209
retina	133
fetal lung	1409
fetal lung fibroblast IMR-90 cells	989
fetal lung fibroblast MRC-5 cells	391
lung	234
lung right upper lobe	153
lung right mid lobe	97
lung right lower lobe	182
lung lupus disease	50
lung tumor	241
lung COPD	43
trachea	471

Tissue	Relative Expression
cervix	36
testis	161
HeLa cells (cervix tumor)	186
placenta	471
uterus	91
uterus tumor	942
ovary	176
ovary tumor	1046
breast	355
breast tumor	286
MDA MB 231 cells (breast tumor)	671
mammary gland	156
prostate	458
prostate BPH	27
bladder	141
ureter	335
penis	162
corpus cavernosum	28
fetal kidney	699
kidney	545
kidney tumor	148
HEK 293 cells	617

REFERENCES

cDNA cloning and expression of a novel family of enzymes with calcium-independent phospholipase A2 and lysophospholipase activities. Portilla D, *et al.*, J Am Soc Nephrol 1998 Jul;9(7):1178-86.

Crystal structure of carboxylesterase from *Pseudomonas fluorescens*, an alpha/beta hydrolase with broad substrate specificity, Kim KK, *et al.*, Structure 1997 Dec 15;5(12):1571-84.

CLAIMS

1. An isolated polynucleotide being selected from the group consisting of:

a. a polynucleotide encoding an esterase polypeptide comprising an amino acid sequence selected form the group consisting of:

i. amino acid sequences which are at least about 97% identical to the amino acid sequence shown in SEQ ID NO: 2; and

ii. the amino acid sequence shown in SEQ ID NO: 2.

b. a polynucleotide comprising the sequence of SEQ ID NO: 1;

c. a polynucleotide which hybridizes under stringent conditions to a polynucleotide specified in (a) and (b) and encodes a esterase polypeptide;

d. a polynucleotide the sequence of which deviates from the polynucleotide sequences specified in (a) to (c) due to the degeneration of the genetic code and encodes a esterase polypeptide; and

e. a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d) and encodes a esterase polypeptide.

2. An expression vector containing any polynucleotide of claim 1.

3. A host cell containing the expression vector of claim 2.

4. A substantially purified esterase polypeptide encoded by a polynucleotide of claim 1.

5. A method for producing a esterase polypeptide, wherein the method comprises the following steps:
   a. culturing the host cell of claim 3 under conditions suitable for the expression of the esterase polypeptide; and
   b. recovering the esterase polypeptide from the host cell culture.

6. A method for detection of a polynucleotide encoding a esterase polypeptide in a biological sample comprising the following steps:
   a. hybridizing any polynucleotide of claim 1 to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and
   b. detecting said hybridization complex.

7. The method of claim 6, wherein before hybridization, the nucleic acid material of the biological sample is amplified.

8. A method for the detection of a polynucleotide of claim 1 or a esterase polypeptide of claim 4 comprising the steps of:
   a. contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the esterase polypeptide and
   b. detecting the interaction

9. A diagnostic kit for conducting the method of any one of claims 6 to 8.

10. A method of screening for agents which decrease the activity of a esterase, comprising the steps of:
    a. contacting a test compound with any esterase polypeptide encoded by any polynucleotide of claim1;
    b. detecting binding of the test compound to the esterase polypeptide, wherein a test compound which binds to the polypeptide is identified

as a potential therapeutic agent for decreasing the activity of a esterase.

11. A method of screening for agents which regulate the activity of a esterase, comprising the steps of:

a. contacting a test compound with a esterase polypeptide encoded by any polynucleotide of claim 1; and

b. detecting a esterase activity of the polypeptide, wherein a test compound which increases the esterase activity is identified as a potential therapeutic agent for increasing the activity of the esterase, and wherein a test compound which decreases the esterase activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the esterase.

12. A method of screening for agents which decrease the activity of a esterase, comprising the step of:

contacting a test compound with any polynucleotide of claim 1 and detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of esterase.

13. A method of reducing the activity of esterase, comprising the step of:

contacting a cell with a reagent which specifically binds to any polynucleotide of claim 1 or any esterase polypeptide of claim 4, whereby the activity of esterase is reduced.

14. A reagent that modulates the activity of a esterase polypeptide or a polynucleotide wherein said reagent is identified by the method of any of the claim 10 to 12.

15. A pharmaceutical composition, comprising:
the expression vector of claim 2 or the reagent of claim 14 and a pharmaceutically acceptable carrier.

16. Use of the expression vector of claim 2 or the reagent of claim 14 in the preparation of a medicament for modulating the activity of a esterase in a disease.

17. Use of claim 16 wherein the disease is cancer, a dermatological disorder, a gastrointestinal and a liver disorder, a neurological disorder, a respiratory disorder, a metabolic disorder, an inflammatory disorder, a reproductive disorder or a genitourinary disorder.

Fig. 1

```
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tgctgcccgc cctgccccgag ccgcatcgcc gccaagctgg ccttcctgcc gcccgagccc     120
acctacacgg tgctggcgcc ggagcagcgc ggcgccggcg cgtccgcccc ggccccggcc     180
caggctaccg ccgccgccgc cgcggcccag ccggcaccgc agcagcccga ggagggcgcg     240
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ttctcccatg gcctagcgat gtacgagcgc tgtccccgag ccgtggagcc cctttgggtt     900
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ttcatatctc acgaacttcc taattcc                                        987
```

Fig. 2

```
Met Pro Glu Pro Gly Pro Arg Met Asn Gly Phe Ser Leu Gly Glu Leu
 1               5                  10                  15
Cys Trp Leu Phe Cys Cys Pro Pro Cys Pro Ser Arg Ile Ala Ala Lys
            20                  25                  30
Leu Ala Phe Leu Pro Pro Glu Pro Thr Tyr Thr Val Leu Ala Pro Glu
        35                  40                  45
Gln Arg Gly Ala Gly Ala Ser Ala Pro Ala Pro Ala Gln Ala Thr Ala
    50                  55                  60
Ala Ala Ala Ala Ala Gln Pro Ala Pro Gln Gln Pro Glu Glu Gly Ala
65                  70                  75                  80
Gly Ala Gly Pro Gly Ala Cys Ser Leu His Leu Ser Glu Arg Ala Asp
                85                  90                  95
Trp Gln Tyr Ser Gln Arg Glu Leu Asp Ala Val Glu Val Phe Phe Ser
            100                 105                 110
Arg Thr Ala Arg Asp Asn Arg Leu Gly Cys Met Phe Val Arg Cys Ala
        115                 120                 125
Pro Ser Ser Arg Tyr Thr Leu Leu Phe Ser His Gly Asn Ala Val Asp
    130                 135                 140
Leu Gly Gln Met Cys Ser Phe Tyr Ile Gly Leu Gly Ser Arg Ile Asn
145                 150                 155                 160
Cys Asn Ile Phe Ser Tyr Asp Tyr Ser Gly Tyr Gly Val Ser Ser Gly
                165                 170                 175
Lys Pro Ser Glu Lys Asn Leu Tyr Ala Asp Ile Asp Ala Ala Trp Gln
            180                 185                 190
Ala Leu Arg Thr Arg Tyr Gly Val Ser Pro Glu Asn Ile Ile Leu Tyr
        195                 200                 205
Gly Gln Ser Ile Gly Thr Val Pro Thr Val Asp Leu Ala Ser Arg Tyr
    210                 215                 220
```

Fig. 2 (continued)

```
Glu Cys Ala Ala Val Ile Leu His Ser Pro Leu Met Ser Gly Leu Arg
225                 230                 235                 240
Val Ala Phe Pro Asp Thr Arg Lys Thr Tyr Cys Phe Asp Ala Phe Pro
                245                 250                 255
Ser Ile Asp Lys Ile Ser Lys Val Thr Ser Pro Val Leu Val Ile His
            260                 265                 270
Gly Thr Glu Asp Glu Val Ile Asp Phe Ser His Gly Leu Ala Met Tyr
        275                 280                 285
Glu Arg Cys Pro Arg Ala Val Glu Pro Leu Trp Val Glu Gly Ala Gly
    290                 295                 300
His Asn Asp Ile Glu Leu Tyr Ala Gln Tyr Leu Glu Arg Leu Lys Gln
305                 310                 315
Phe Ile Ser His Glu Leu Pro Asn Ser
                325
```

Fig. 3

```
ctccctcgcc gcgcgtccgt cctccgtcct cccgggccag ccagccagcc agccagccgg      60
gccggcggcg ggcaccaggc cgtcccgatg cccgagccag gccccaggat gaacggcttc     120
tcgctgggtg agctgtgctg gctcttctgc tgcccgccct gcccgagccg catcgccgcc     180
aagctggcct tcctgccgcc cgagcccacc tacacggtgc tggcgccgga gcagcgcggc     240
gccggcgcgt ccgccccggc cccggcccag gctaccgccg ccgccgccgc ggcccagccg     300
gcaccgcagc agcccgagga gggcgcgggc gcggggcccg gtgcgtgcag cctgcacctc     360
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tcgcgcacgg cccgggacaa ccggctcggc tgcatgttcg tgcgctgcgc gccctccagc     480
cgctacacgc tgctcttctc gcacggcaac gccgtggacc tgggccagat gtgcagcttc     540
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ggcgtcagct cgggcaagcc ctccgagaag aacctctacg ccgacatcga cgccgcgtgg     660
caggcgctgc gcacccggta tggcgtgagt cccgagaaca ttatcctcta tggtcagagc     720
attgggactg tccccacggt agacttggcc tcgaggtatg aatgcgcagc ggtaattctc     780
cattcccctc tgatgtctgg tttgcgtgtg gcttttccgg ataccaggaa aacatactgc     840
tttgatgctt tccccagcat tgacaagata tctaaagtca cctctcctgt gttggtcatt     900
catggtacag aggatgaggt catcgatttc tcccatggcc tagcgatgta cgagcgctgt     960
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gcacaatacc tagaaagact aaaacagttc atatctcacg aacttcctaa ttcctgaaga    1080
caacaacttg atcttacctc atttactgtg aacagaagag tcctctgttt tgcacatgct    1140
ttaactgggt agctgtaaag gcttgataac catgaagaag tgcccaacct ttagggtgtt    1200
ctaatcaaag agctgatgaa atctcagtct tttgtatcta gaggtggttc tgctaattca    1260
cacaacacgt taaactgaac agtcgtgatt cccagcttca ttaccttgca ggaatgggaa    1320
tgagagctga atgtagggac aattttctag tgctgtataa agtagcctcg catctgtttc    1380
tcaaccttat ccatcatttc tgacattcat gcaggacttg ccctgttgcc accaatgttc    1440
tcggtatttc acatgcagct ctctttctgc cactggatac atgggttcaa tccatttgtg    1500
aagctgtgat agtgtaactg gaaagctagt gtggtgaaaa ttcctttatt atttttgtt     1560
aacatgctga tctttcccgg acaaatgaac tgaagggtaa tttactggaa ctctcgtgta    1620
cagcttcatc aactgtaacc atataaatat aactggaata ttcttaaaca aaaagaaact    1680
```

Fig. 3 (continued)

```
aggggttttt ttaagtgtaa atttattact agccaacaga gttttactat tttgattgtc  1740
tggttggttt aacaaagagc ctagctgact ttccttctgt aaagtcctcc ttgtaggctt  1800
ttttaaagta ctgtacatat ttgcaatcac attgtgcata gattcttaat ggtagatatg  1860
atttcttttg tcaggctaca acaatgaact gcagattcct tgtttgtaat gtaaatgatt  1920
gaatacattt tgttaatatg tttttattcc tatgttttgc tattaaaaat tttataacat  1980
ttccaagaca aaaattccaa gtttatgctt tgaagaattt atgtaattaa aatttcacta  2040
aactaatctt tttagtttag gaattatttg ggttttgaca ctggaagttg cgccaaataa  2100
gcatcagaaa taggagatgc ttaacattgc tatactactt gtgttggtta ggggtttgga  2160
tttgggggtt ctttggtttt aatttttttt tccacattta aaagccttaa atgtactgta  2220
agcctcagat cgttgtacaa ctggactgcg gttgattgcc agtttgtgta ctgttgcttg  2280
gatgcggcac agtggttggt aatggaataa aggatgcatg gatcag                 2326
```

Fig. 4

```
gggcccggtg cgtgcagcct gcacctcagc gagcgcgccg actggcagta ctcgcagcgc    60
gagctggacg ccgtcgaggt cttcttctcg cgcacggccc gggacaaccg gctcggctgc   120
atgttcgtgc gctgcgcgcc ctccagccgc tacacgctgc tcttctcgca cggcaacgcc   180
gtggacctgg gccagatgtg cagcttctac attggcctcg gctcccgcat caactgcaac   240
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gagaacatta tcctctatgg tcagagcatt gggactgtcc ccacggtaga cttggcctcg   420
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aaagtcacct ctcctgtgtn ggtcattcat ggtacagagg atgaggtcat cgatttctcc   600
catggcctag cgatgtacga gcgctgtccc cgagccgtgg agcccctttg ggttgaaggg   660
gctgggcata atgacataga gctttatgca caatacctag aaagactaaa acagttcata   720
tctcacgaac ttccta                                                   736
```

Fig. 5

```
tcagccctcc gagaagaacc tctacgccga catcgacgcc gcgtggcagg cgctgcgcac      60
ccggtatggc gtgagtcccg agaacattat cctctatggt cagagcattg ggactgtccc     120
cacggtagac ttggcctcga ggtatgaatg cgcagcggta attctccatt cccctctgat     180
gtctggtttg cgtgtggctt ttccggatac caggaaaaca tactgctttg atgctttccc     240
cagcattgac aagatatcta aagtcacctc tcctgtgttg gtcattcatg gtacagagga     300
tgaggtcatc gatttctccc atggcctagc gatgtacgag cgctgtcccc gagccgtgga     360
gccccttggg gttgaagggg ctgggcataa tgacatagag cttta                     405
```

Fig. 6

```
cacgcgtccg agagcattgg gactgtcccc acggtagact tggcctcgag gtatgaatgc      60
gcagcggtaa ttctccattc ccctctgatg tctggtttgc gtgtggcttt tccggatacc     120
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gacatagagc tttatgcaca atacctagaa agactaaaac agttcatatc tcacgaactt     360
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ggttctgcta attcacacaa cacgttaaac tgaacagtcg tgat                      584
```

Fig. 7

```
cgtgtggctt ttccggatac caggaaaaca tactgctttg atgctttccc cagcattgac     60
aagatatcta aagtcacctc tcctgtgttg gtcattcatg gtacagagga tgaggtcatc    120
gatttctccc atggcctagc gatgtacgag cgctgtcccc gagccgtgga gccccttggg    180
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actgtgaaca gaagagtcct ctgttttgca catgctttaa ctgggtagct gtaaaggctt    360
gataaccatg aagaagtgcc caacctttag ggtgttctaa tc                       402
```

Fig. 8

```
gcgtgtggct tttccggnta ccaggaaaac atactgcttt gatgctttcc ccagcattga     60
caagatatct aaagtcacct ctcctgtgtt ggtcattcat ggtacagagg atgaggtcat    120
cgatttctcc catggcctag cgatgtacga gcgctgtccc cgagccgtgg agccccttg     180
ggttgaaggg gctgggcata atgacataga gctttatgca caatacctag aaagactaaa    240
acagttcata tctcacggac ttcctaattc ctgaaggcaa caacttgatc ttacct        296
```

Fig. 9

```
cgtgtggctt ttccggatac caggaaaaca tactgctttg atgctttccc cagcattgac     60
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actgtgaaca gaagagtcct ctgttttgca catgctttaa ctgggtagct gtaaaggctt    360
gataaccatg gaagaagtgc ccaaccttta gggtgttcnt aatcaaagag ctggatgg      418
```

Fig. 10

```
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acttgccctg ttgccaccaa tgttctcggt atttcacatg cagctctctt tctgccactg    660
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```

Fig. 11

```
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35 40 45
Gln Arg Gly Ala Gly Ala Ser Ala Pro Ala Pro Ala Gln Ala Thr Ala
50 55 60
Ala Ala Ala Ala Ala Gln Pro Ala Pro Gln Gln Pro Glu Glu Gly Ala
65 70 75 80
Gly Ala Gly Pro Gly Ala Cys Ser Leu His Leu Ser Glu Arg Ala Asp
85 90 95
Trp Gln Tyr Ser Gln Arg Glu Leu Asp Ala Val Glu Val Phe Phe Ser
100 105 110
Arg Thr Ala Arg Asp Asn Arg Leu Gly Cys Met Phe Val Arg Cys Ala
115 120 125
Pro Ser Ser Arg Tyr Thr Leu Leu Phe Ser His Gly Asn Ala Val Asp
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Cys Asn Ile Phe Ser Tyr Asp Tyr Ser Gly Tyr Gly Val Ser Ser Gly
165 170 175
Lys Pro Ser Glu Lys Asn Leu Tyr Ala Asp Ile Asp Ala Ala Trp Gln

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Gly Gln Ser Ile Gly Thr Val Pro Thr Val Asp Leu Ala Ser Arg Tyr
    210                 215                 220
Glu Cys Ala Ala Val Ile Leu His Ser Pro Leu Met Ser Gly Leu Arg
225                 230                 235                 240
Val Ala Phe Pro Asp Thr Arg Lys Thr Tyr Cys Phe Asp Ala Phe Pro
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Ser Ile Asp Lys Ile Ser Lys Val Thr Ser Pro Val Leu Val Ile His
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Gly Thr Glu Asp Glu Val Ile Asp Phe Ser His Gly Leu Ala Met Tyr
        275                 280                 285
Glu Arg Cys Pro Arg Ala Val Glu Pro Leu Trp Val Glu Gly Ala Gly
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<211> 418
<212> DNA
<213> Homo sapiens
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<222> (182)..(182)
<223> n = a, t, g, c
<220>
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<222> (399)..(399)
<223> n = a, t, g, c
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<211> 712
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<213> Homo sapiens
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<213> Homo sapiens
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